Proceedings
of the
Society
for
Experimental Biology and Medicine

Vol. 83 August-September, 1953 No. 4

Eosinopenic Responses of Pyridoxine-Deficient Rats to Epinephrine, Adrenocorticotropic Hormone and Hypoxia.* (20448)

Lillian C. Butler† and Agnes Fay Morgan.
From the Department of Home Economics, University of California, Berkeley.

Few studies of the adrenal or pituitary function of the pyridoxine-deficient rat have been published. Histological studies of Dean and Shaw(1) showed at 3 weeks no more than a mild transitory stimulation of the zona fasciculata and reticularis of the pyridoxine-deficient rat. In 6 to 10 weeks this disappeared and the gland did not differ from that of the control. More recently, Stebbins(2) reported profound changes in the adrenal of the pyridoxine-deficient rat. At weekly intervals during a period of 5 weeks progressive depletion of the sudanophilic lipoid, birefringent material, cholesterol and ascorbic acid occurred in the fasciculata. These adrenal alterations were not present in the inanition and *ad libitum* controls. Impaired water diuresis which was restored to normal with desoxycorticosterone acetate (DOCA), lipo-adrenal extract or pyridoxine was also observed by Stebbins(3) in the pyridoxine-deficient rat. Ershoff and Parrott(4) found a normal lymphopenic response to a single dose of epinephrine in the pyridoxine-deficient rat. When epinephrine was injected daily for 10 successive days, however, an impaired lymphopenic response resulted. Whether this was caused by the deficiency itself or by the effects of inanition, these workers were not able to ascertain because caloric restricted rats were not studied.

The present study was restricted to one technic of determining the functional capacity of the pituitary and adrenal of the pyridoxine-deficient rat, that measured by eosinopenia. Both epinephrine and adrenocorticotropic hormone (ACTH) were used by Thorn(5) in eosinopenic tests for the diagnosis of hypopituitarism and adrenal insufficiency in humans. In addition, hypoxia has been well established as a stimulus of the pituitary adrenal system(6). Low barometric pressure causes specific metabolic changes in the intact rat(7) but not in the adrenalectomized rat(8).

Methods and materials. Diets and animals. Normal adult male Long-Evans rats with a mean weight of 376 g at a mean age of 103 days were placed in individual metabolism cages. Three groups were established; deficient, pair weighed, and those fed *ad libitum*. For 8 weeks all received a basal diet consisting of 24% casein (Labco, vitamin tested), 4% Hubbell *et al.*(9) salt mix and 62% sucrose. Vitamins‡ were fed 3 times weekly in individual cups. The pyridoxine-deficient group received 30 mg of desoxypyridoxine per kg diet mixed directly into the ration, and pyridoxine was omitted from their vitamin supplements. The average weight loss in the deficient and pair weighed groups was 29%, while the *ad libitum* group gained 1%. In the deficient group mild acrodynia of the nose and fore paws in the 4th week progressed during the 7th and 8th weeks into severe involvement of the nose and all paws accompanied by alopecia.

---

* The authors wish to express their appreciation to Dr. William O. Reinhardt and Dr. Gerald F. Hungerford for suggestions and aid. We also wish to thank Dr. Choh Hao Li, University of California, Berkeley, for the ACTH preparation and Dr. Gladys A. Emerson of Merck and Co., Rahway, N. J. for the desoxypyridoxine.

† Public Health Service Research Fellow of the National Institute of Arthritis and Metabolic Diseases.

Initial eosinophil counts were made immediately prior to the injections and 4 hours later. Thirty rats of *Series I* were injected during the 7th week on consecutive days with saline, 25 μg of epinephrine and 1 mg of ACTH. During the 8th week of the regimen these same animals were hypophysectomized. Twenty-four hours postoperatively 1 mg of ACTH was administered (Table I).

Series II comprising 48 rats was studied in the 4th and 7th weeks of the regimen for the purpose of determining any possible transitory eosinopenic response changes. Twenty-four rats received injections of 50 μg of epinephrine in these 2 periods and one injection of saline in the 4th week (Table I). In the 8th week of this dietary regimen the 24 rats of Series II were subjected to 24 hours of fasting and during the last 20 hours to hypoxia(10) equal to a decreased atmospheric pressure of 349 mm of mercury equivalent to 20,000 ft altitude. The animals were individually confined in 2-qt Mason jars which were attached to the low barometric pressure system. The animals had free access to water. The initial eosinophil counts used in this experiment were determined 3 days prior to subjecting the animals to hypoxia. The final eosinophil counts were completed immediately after the pressure was gradually increased to sea level. In like manner 24 rats of Series II in the 8th week of the regimen were fasted 24 hours and were placed into 2-qt jars during the last 20 hours of the 24-hour fasting period. These jars were also placed within the confines of the apparatus without connecting them to the hypoxia system. Eosinophil counts were similarly determined (Table I). The animals of Series III received saline injections followed by epinephrine injections 24 hours later during the 8th week of the regimen. This series consisted of 5 deficient and 5 pair weighed animals (Table I).

Blood technic and injections. Because diurnal variation(11) is an important factor in obtaining a constant eosinophil count from day to day all 4-hour tests began at 9:00 A.M. and were completed by 3:30 P.M. Insofar as possible environmental conditions were standardized(12). The animals were not disturbed 12 hours prior to the counts(13). Free flowing tail vein blood was obtained within 2 minutes after removing the animals from the cage and duplicate white blood pipettes were filled to the 0.5 mark. One chamber of a 0.2 mm Fuchs-Rosenthal hemocytometer was counted for each pipette. The diluent was prepared according to Speirs and Meyer(13). The per cent eosinophil change was statistically evaluated as a plus or minus value. The aqueous solution of 0.5 ml of 0.9% sodium chloride was administered intraperitoneally. Epinephrine hydrochloride (Adrenaline hydrochloride, Parke Davis & Co.) was diluted with 0.9% sodium chloride to contain either 50 μg or 25 μg per 0.5 ml. This solution was administered subcutaneously in 0.5 ml quantities. In the intact rat Hungerford(14) demonstrated graded eosinopenic responses to epinephrine in doses from 10 μg to 150 μg provided it was administered subcutaneously rather than intraperitoneally. A solution of 1 mg ACTH prepared from sheep pituitary glands(15) in 0.5 ml of 0.9% sodium chloride solution was administered intraperitoneally. A 0.5 ml syringe and a size 24 needle were used for all injections.

Results. Initial circulating eosinophil levels. The initial circulating eosinophil levels in Series I, II and III showed no differences

---

‡ The water soluble vitamins were dissolved in 20% aqueous ethanol solution and supplied each rat daily the following amounts in mg: thiamine hydrochloride, riboflavin, pyridoxine, and folic acid, .02 each, Ca pantothenate and para-aminobenzoic acid, .10 each, niacin amide, .07, inositol, 2.5, biotin, .002, and choline, 5.0. Vits. A and D and mixed tocopherols were dissolved in vegetable oil and were supplied separately in amounts of 1000 I. U., 100 I. U. and 3 mg/rat/week.

TABLE I. Eosinopenic Responses of the Pyridoxine-Deficient Rat Induced by Saline, Epinephrine, ACTH, Fasting and Hypoxia.

Series	Treatment	Week	No. of rats	Group	Eosinophils/mm Initial	Eosinophils/mm Final	% change†	P*
I. Intact	Saline .5 ml	7	10	D§	415 ± 60	275 ± 76	—41 ± 9	
			9	PW	369 ± 91	202 ± 79	—52 ± 10	
			10	AL	478 ± 81	313 ± 46	—40 ± 6	
	Epinephrine 25 μg	7	10	D	454 ± 89	108 ± 22	—79 ± 3	<.01
			10	PW	388 ± 38	167 ± 74	—62 ± 6	<.30
			10	AL	519 ± 69	213 ± 44	—59 ± 7	.07
	ACTH 1 mg	7	10	D	513 ± 60	167 ± 78	—73 ± 7	.02
			10	PW	382 ± 72	141 ± 46	—65 ± 8	<.20
			10	AL	514 ± 50	70 ± 62	—68 ± 6	<.01
Hypophysectomized	ACTH 1 mg	8	7	D	401 ± 111	152 ± 62	—66 ± 8	
			7	PW	559 ± 67	247 ± 78	—65 ± 11	
			7	AL	922 ± 225	388 ± 171	—60 ± 8	
II. Intact	Saline .5 ml	4	8	D	498 ± 144	426 ± 79	+ 2 ± 6	
			8	PW	621 ± 199	337 ± 70	—47 ± 8	
			8	AL	454 ± 92	385 ± 46	—19 ± 11	
	Epinephrine 50 μg	4	8	D	514 ± 136	93 ± 25	—76 ± 7	<.01
			8	PW	424 ± 128	134 ± 10	—83 ± 8	<.01
			8	AL	454 ± 38	83 ± 13	—80 ± 5	<.01
	Epinephrine 50 μg	7	8	D	374 ± 12	77 ± 24	—78 ± 6	<.01
			8	PW	297 ± 24	79 ± 24	—75 ± 8	<.01
			8	AL	529 ± 46	53 ± 8	—88 ± 3	<.01
	Sea level fasted	8	8	D	403 ± 29	170 ± 34	—55 ± 10	
			8	PW	364 ± 97	115 ± 32	—52 ± 13	
			8	AL	419 ± 35	284 ± 56	—31 ± 17	
	Hypoxia 20000 ft	8	8	D	370 ± 38	7 ± 4	—98 ± 1	<.01‡
			8	PW	297 ± 68	21 ± 9	—89 ± 6	.03
			8	AL	476 ± 120	25 ± 9	—93 ± 3	<.01
III. Intact	Saline .5 ml	8	5	D	514 ± 156	423 ± 145	—14 ± 14	
			5	PW	654 ± 17	250 ± 18	—60 ± 8	
	Epinephrine 50 μg	8	5	D	504 ± 145	114 ± 59	—86 ± 9	<.01
			5	PW	405 ± 90	46 ± 20	—91 ± 3	<.01

* Probability of significance of the % change of each group compared with its respective eosinopenia induced by saline injection.

† $\% \text{ change} = 100 \times \dfrac{\text{Initial} - \text{final}}{\text{Initial}}$.

‡ Probability of significance of the % change of each group compared with its respective % change at sea level.

§ D = deficient, PW = pair weighed, AL = *ad libitum.*

within each group from day to day or within the same week on the dietary regimen (Table I). The hypophysectomized animals showed no differences from their respective unoperated groups. However, in Series II during the 7th week and 8th week the initial circulating eosinophil levels of the deficient and pair weighed groups were significantly decreased when compared with their respective initial eosinophil levels during the 4th week.

Series I. Eosinopenias induced by saline, epinephrine and ACTH. During the 7th week the decrease in the eosinophils of all 3 groups following saline injection was —40% to —50% (Table I). This change was of the same magnitude in each group. Epinephrine injected at the 25 μg dose level gave a significant change in the deficient group when compared with its own saline-induced eosinopenia. Obviously, 25 μg of epinephrine was not large enough a dose to produce a significant response either in the pair weighed group or in the *ad libitum* group. ACTH caused a significant eosinopenia in the unoperated animals of the deficient group (—73%) and of the *ad libitum* group (—65%) as compared with their respective saline responses. When these previously mentioned responses to ACTH are compared with each other, all 3 groups exhibited the same degree of eosinopenia. The 3 hypophysectomized groups showed equal eosinopenic responses to ACTH which did not differ from their corresponding ACTH-induced eosinopenias before the operation.

Series II. Eosinopenias induced by saline and epinephrine. Saline caused an eosinophilia of +2% during the 4th week in the deficient group. This value differed significantly from its corresponding deficient group (—41%) during the 7th week in Series I but not from the deficient group (—14%) in Series III in the 8th week. No differences in the saline-induced eosinopenia in the pair weighed (—47%) and *ad libitum* group (—19%) occurred. The degree of eosinopenia resulting from the administration of 50 μg of epinephrine during the 4th week and 7th week was comparable (—75% to —88%) in all 3 groups, and all were significantly below their respective saline eosinopenias.

Series II. Eosinopenias induced by fasting and hypoxia. The changes at sea level of the fasted animals reflected the stresses of handling, fasting 24 hours and of confinement in the hypoxia chamber for the last 20 hours. The eosinopenia in all 3 groups was of the same magnitude (—31% to —55%, Table I). In all 3 groups hypoxia caused comparable eosinopenias (—89% to —93%) which were significantly greater than the eosinopenic responses of their respective groups at sea level.

Series III. Eosinopenias induced by saline and epinephrine. During the 8th week a separate series of animals was studied for the purpose of confirming our previous results with epinephrine. The eosinopenia caused by saline was significantly larger in the pair weighed group (—60%) than in the deficient group (—14%). Administration of 50 μg of epinephrine again caused a significant eosinopenia in both the deficient and pair weighed groups (—86% and —91%, respectively) as compared with their saline-induced responses.

Discussion. The present experiment demonstrates that the pyridoxine-deficient rat was capable of responding to stress with a significant eosinopenia as well as did the pair weighed and *ad libitum* controls. It may be assumed therefore, that there was no interference in the elaboration or release of ACTH and adrenal 11-oxysteroids in the pyridoxine-deficient rat as determined by the epinephrine tests employed during the 4th week of mild deficiency signs and during the 7th and 8th weeks when severe acrodynia was present. It has generally been found that epinephrine acts directly upon the pituitary of rats causing the release of ACTH(16-19). Gemzell(19) in addition showed a fall of the pituitary ACTH content and a concomitant rise in the body fluid ACTH content in rats. In humans this action has not been demonstrated(20). When the pituitary adrenal axis was interrupted in rats by hypophysectomy or adrenalectomy, it has been established that no eosinopenic response followed the administration of a physiological dose of epinephrine(13,16,17). On the other hand, a synergistic action of epinephrine with cortisone has been demonstrated(21,22). When either substance alone is incapable of producing an eosinopenia at the dose levels used, evidence of their combined eosinopenic efficaciousness has been found both in adrenalectomized humans by Kark and Muehrcke(21) and adrenalectomized dogs by Henry *et al.*(22).

The use of ACTH-induced eosinopenia as a test for the adrenal function has been reported to be reliable for humans(21,23,24) and for rats and dogs(13,15). The data show that ACTH in the intact rat gave a significant eosinopenia in the pyridoxine-deficient and *ad libitum* groups but not in the pair weighed group. This was probably due to the greater saline-induced eosinopenia (by —11%) of this latter group. In order to obviate any activity mediated by the pituitary, hypophysectomized rats were used. The use of the hypophysectomized animal has the advantage of offering a more valid method of testing the adrenal function because endogenous ACTH is not present. All 3 of these hypophysectomized groups including the pyridoxine-deficient group gave eosinopenic responses greater than —50%. Our data made it evident that the adrenal cortex of the hypophysectomized pyridoxine-deficient rats is capable of normal function as determined by the eosinopenia induced by ACTH administration.

Hypoxia was shown to cause eosinopenia in dogs by Furgeson and Smith(25) and in rats during recovery from hypoxia by King *et al.* (26). Our experiments demonstrate that the pyridoxine-deficient rat is capable of a normal eosinopenic response following 20 hours of exposure to an altitude of 20,000 ft including a 24-hour fast. Fasting of rats confined in the hypoxia chamber at sea level caused sufficient stress to produce a sharp drop in the circulating eosinophils. The latter finding suggests that eosinopenia is a more sensitive index of alterations in the pituitary and adrenal activity than are metabolic and adrenal changes (27) which fail to occur under these fasting conditions in the normal rat(28).

The differences between the adrenal histological findings of Stebbins(2) and Deane and Shaw(1) may be attributed to unequal susceptibility of dissimilar strains of rats to pyridoxine-deficiency(29); the former used Carworth male weanlings while the latter used Long-Evans young male rats. The current study of the adult pyridoxine-deficient male Long-Evans rat given desoxypyridoxine did not duplicate either of the previously mentioned experimental conditions. The eosinopenic responses in the adult pyridoxine-deficient male rat during the 4th, 7th, and 8th weeks indicated a normal response of the pituitary adrenal system to the stimuli used. Extra-adrenal factors may well be involved in eosinopenia but adrenal hormones appear to have at least an indirect influence.

Summary. Eosinopenic responses following, 1) hypoxia treatment, and 2) epinephrine injections, both in the intact rat, and 3) ACTH injections in the intact and in the hypophysectomized rat were of equal magnitude in the pyridoxine-deficient, pair weighed and normal animals. The significance of these findings is discussed.

---

1. Dean, H. W., and Shaw, J. H., *J. Nutr.*, 1947, v34, 1.

2. Stebbins, R. B., *Endocrinology,* 1950, v49, 25.

3. ———, *Am. J. Physiol.,* 1950, v106, 538.

4. Ershoff, B. H., and Parrott, F. D., *J. Clin. Nutr.*, 1953, v1, 124.

5. Thorn, G. W., *Diagnosis and Treatment of Adrenal Insufficiency,* pp. 53-61, 2nd Ed., 1951, Charles C. Thomas, Springfield.

6. Sayers, G., *Physiol. Rev.,* 1950, v30, 241.

7. Tepperman, J., Tepperman, H. M., Patton, B. W., and Nims, L. F., *Endocrinology,* 1947, v41, 356.

8. Lewis, R. A., Thorn, G. W., Koepf, G. F., and Dorrance, S. S., *J. Clin. Invest.,* 1942, v21, 33.

9. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutr.,* 1937, v14, 273.

10. Wickson, M. E., and Morgan, A. F., *J. Biol. Chem.,* 1946, v162, 209.

11. Halberg, F., and Visscher, M. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 846.

12. Halberg, F., Bittner, J. J., and Visscher, M. B., *Blood,* 1951, v6, 832.

13. Speirs, R. S., and Meyer, R. K., *Endocrinology,* 1949, v45, 403.

14. Hungerford, G. F., Unpublished data.

15. Li, Choh Hao, *J. Am. Chem. Soc.,* 1952, v74, 2124.

16. Recant, L., Hume, D. H., Forsham, P. H., and Thorn, G. W., *J. Clin. Endoc.,* 1950, v10, 187.

17. McDermott, W. V., Fry, E. G., Brobeck, J. R., and Long, C. N. H., *Yale J. Biol. and Med.,* 1950, v23, 52.

18. Gershberg, H., Fry, E. G., Brobeck, J. R., and Long, C. N. H., *Yale J. Biol. and Med.,* 1950, v23, 32.

19. Gemzell, C. A., *Endocrinology,* 1952, v50, 399.

20. Thorn, G. W., *Am. J. Med.,* 1953, v24, 139.

21. Kark, R. M., and Muehrcke, R. C., *Lancet,* 1952, v22, 1189.

22. Henry, W. L., Oliner, L., and Ramey, E. R., *Fed. Proc.,* 1953, v12, 66.

23. Thorn, G. W., Forsham, P. H., Prunty, F. T., and Hills, A. G., *J. Am. Med. Assn.,* 1948, v137, 1005.

24. Best, W. R., Muehrcke, R. C., and Kark, R. M., *J. Clin. Med.,* 1951, v38, 790.

25. Furgeson, F. P., and Smith, D. C., *Fed. Proc.,* 1953, v12, 42.

26. King, C. T. G., Mego, J. L., Williams, E. E., and Shaeffer, K. E., *Fed. Proc.,* 1953, v12, 76.

27. Gordon, A. S., and Padawer, J., *Fed. Proc.,* 1953, v12, 54.

28. Hurley, L. S., and Morgan, A. F., *J. Biol. Chem.,* 1952, v195, 583.

29. Cerecedo, L. R., and Renzo, E. C., *Arch. Biochem.,* 1948, v17, 397.

Received May 4, 1953. P.S.E.B.M., 1953, v83.

Effect of Acute Cold Exposure on Serum Potassium, and Magnesium and the Electrocardiogram in Man. (20449)

MURRAY QUINN, DAVID E. BASS, AND CHARLES R. KLEEMAN. (Introduced by A. Henschel)

From the Biochemistry Branch, Quartermaster Climatic Research Laboratory, Lawrence, Mass.

During exposure to low environmental temperatures metabolic changes involving potassium and magnesium occur. Increases in the levels of potassium in serum have been described in animals subjected to acute cold exposure(1) and in men transferred from a warm climate to an arctic environment(2). Magnesium has been implicated as an important factor in temperature regulation primarily on the basis of animal experiments, but the data in man are scanty and controversial(3). Hypothermic rats develop increased cardiac sensitivity to injected potassium salts(4); however, the electrocardiographic changes described in hypothermia(5) were not entirely characteristic of the changes observed in either hyperkalemia(6) or hypermagnesemia(7).

The present study was undertaken to determine the extent and rapidity of any changes that might occur in the concentrations of serum potassium and magnesium during acute cold exposure. Electrolyte changes were also correlated with alteration in serial electrocardiographic patterns.

Materials and methods. Twenty-two experiments were performed on 20 young, normal men. Each subject dressed in underwear reclined for 45 minutes at an ambient temperature of 75°F, then reclined in a cold chamber maintained at 45°-50°F for 90 minutes. Venous blood was obtained without stasis at the end of the control period and at 30-minute intervals in the cold for the determination of magnesium by the method of Simonson, Westover, and Wertman(8), potassium with the Beckman flame photometer, and serum proteins by the copper sulfate specific gravity method. Ionized magnesium was estimated from the total serum magnesium and protein concentrations, using the nomogram of Willis and Sunderman(9). Rectal temperatures were obtained with clinical thermometers at the times that blood samples were drawn. Electrocardiograms were obtained on 19 subjects with a direct writing Sanborn Viso-Cardiette at the end of the control period and after 40 and 80 minutes of cold exposure. The three standard limb leads and V_2 and V_4 were employed. Tests for significance were done by paired data analyses; thus, each individual served as his own control.

Results and discussion. The data are summarized in Table I. The mean serum concentrations of proteins and potassium after 30, 60, and 90 minutes of cold exposure were all statistically significantly higher than the control values ($P < 0.05$). The absence of any correlation between the changes in proteins and potassium ($r = 0.04$, not significant) militates against the possibility that the changes in this electrolyte were due solely to hemoconcentration. There were no statistically significant changes in rectal temperature or concentration of total or ionized serum magnesium. However, any change in the concentration of magnesium may have been masked by hemoconcentration.

Analyses of the electrocardiograms failed to reveal any important changes in cardiac rate, rhythm, or conduction. Examination of the T waves in lead V_4 revealed a significant increase in amplitude after 40 and 80 minutes of cold exposure, but this small increase did not correlate significantly with the increase in concentration of serum potassium.

The mechanism responsible for the increase in the concentration of serum potassium in this study is not clear. Heightened muscle activity may have been partially responsible for this increase since shivering was a prominent feature in this experiment. Such increases have been described in rats during exercise(10) and in man during electroshock therapy(11). The results of the present report confirm the findings in animals subjected to cold exposure(1). Various mechanisms

TABLE I. Mean Values and Standard Deviations of Concentrations of Serum Potassium, and Magnesium and of Height of T Wave in Lead V_4.

Min. of cold exposure	0	30	60	90
Total serum proteins (g/100 cc)	7.24 ± .27	7.47 ± .37	7.62 ± .35	7.75 ± .31
As % of control	100	103	105	107
K (mEq/l)	4.67 ± .30	4.92 ± .30	5.11 ± .30	5.20 ± .36
As % of control	100	105	109	111
Total Mg (mEq/l)	1.55 ± .26	1.57 ± .29	1.61 ± .32	1.53 ± .33
As % of control	100	101	104	99
Ionized Mg (mEq/l)	1.1 ± .2	1.1 ± .1	1.1 ± .2	1.1 ± .2
As % of control	100	100	100	100
		40 min.	80 min.	
ECG lead V_4	8.03	9.22	9.53	
T wave height in mm	± 2.44	± 2.71	± 2.97	
As % of control	100	115	119	

have been postulated to explain the increased serum potassium in the experiments noted, but the nature of the present study does not permit analogous interpretation of the findings reported here.

Summary. Twenty healthy young men exposed to a temperature of 45°-50°F for a period of 90 minutes showed a significant elevation in the mean concentration of serum potassium, but no significant changes in the mean serum concentrations of either total or ionized magnesium. No correlation was observed between the changes in the electrocardiogram and the changes in the concentration of serum potassium.

We are indebted to Miss J. A. Klimas for the statistical analyses. The technical assistance of Mrs. I. T. Dobalian, Mr. A. MacLeod, Pfc R. Bunge, and Pfc J. Lopex is appreciated. We are grateful to Drs. A. Henschel and F. Daniels, Jr. for their suggestions and criticisms.

---

1. Booker, W. M., Hayes, R. L., DaCosta, F. D., Jones, W., Hill, R., and Titus, P., *Fed. Proc.*, 1952, vII, 15.

2. *Survival in the cold:* A metabolic and nutritional study of soldiers acclimatized to heat transported abruptly to a very cold climate. Med. Nutrition Lab. Chicago, Rep. No. 42, Nov. 30, 1948.

3. Hall, V. E., Grant, R. E., and Whalen, W. J., *AF Tech. Rep.* No. 6682, August, 1951.

4. Elliott, H. W., and Crimson, J. M., *Am. J. Physiol.*, 1947, v151, 366.

5. Kossman, C. E., *Bull. N. Y. Academy Med*, 1940, v16, 317.

6. Tarail, R., *Am. J. Med.*, 1948, v5, 828.

7. Smith, P. K., Winkler, A. W., and Hoff, H., *Am. J. Physiol.*, 1939, v126, 720.

8. Simonsen, D. G., Westover, L. M., and Wertman, M., *J. Biol. Chem.*, 1947, v169, 39.

9. Willis, M. J., and Sunderman, F. W., *J. Biol. Chem.*, 1952, v197, 343.

10. Miller, H. C., and Darrow, D. C., *Am. J. Physiol.*, 1941, v132, 801.

11. Welt, L. G., Orloff, J., Kydd, D. M., and Oltman, J. E., *J. Clin. Invest.*, 1950, v29, 935.

Received May 6, 1953. P.S.E.B.M., 1953, v83.

Reproduction in Paramecium as Affected by Small Doses of X-Ray and Beta Radiation. (20450)

George E. Daniel and Helen D. Park. (Introduced by Willie W. Smith)

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md.

During the course of work on the effect of high energy radiation on protozoa it was observed that isolation cultures of *Paramecium caudatum* in a certain group of depression slides consistently produced a larger number of individuals than similar cultures in supposedly identical slides. Chance measurements showed that the former group of slides was slightly radioactive, while the other was not. The active slides were of Czechoslovakian manufacture and had been purchased some 20 years ago. Rough measurements with a G.M. counter held over the slides indicated an activity some 20-30 counts per minute above background. The radiation was rather soft since only 0.040 in. of aluminum was required to reduce the count to background value. These slides had never been used and were still in their original tissue paper wrappers. So far as we know there had been no opportunity for accidental contamination of the slides with radioactive material and, furthermore, neither the wrappers nor a water detergent mixture used to wash the slides showed any activity. The inactive slides were new and of recent American manufacture. Both sets were approximately 75 x 25 mm with depressions 3 mm deep and 15 mm in diameter.

The following observations indicate the degree of differences observed: 1) Five single cell cultures were established in each type of slide using comparable amounts of food and media (lettuce extract). At the end of 24 hours there were 5 paramecia in the inactive slides and 10 in the active ones. 2) Ten isolation cultures were prepared in each type of slide. No transfers were made for 6 days. At the end of this time the active slides had a total population of 2459 paramecia and the inactive slides a total of 1859. 3) Again, 5 cultures in each type of slide were set up and daily examinations were made. A single individual from each slide was transferred to a clean slide of the same type each day, with new media and food. At the end of 7 days the active slides contained 85 paramecia and the inactive 61. There had been two deaths in the active slides and 7 in the inactive. 4) Nine cultures in each type of slide were established and allowed to run for 6 days. At the end of this time there were 238 and 156 paramecia in the active and inactive slides, respectively. The data from the experiments conducted in the radioactive slides are not conclusive because the differences may not be due entirely to the radioactivity of these slides. It may be that the glass liberates into the solution some substance which of itself is stimulating to growth, or such soluble materials may favorably alter the medium. We feel, however, that the difference observed in 4 experiments of 3 different patterns is more than a fortuitous coincidence. It is logical to infer from the above results that rather low concentrations of radiation may have contributed to growth and division. In order to test this hypothesis under more controlled conditions a series of experiments using radiosulfur (S 35) were undertaken.

A clone of *Paramecium caudatum* growing in lettuce extract and fed *Aerobacter aerogenes* was used as a source of experimental material. Experimental and control cultures were set up on the same day from the same stock culture using the same solutions except for the addition of the sulfur to one group. No particular attempt was made to choose stock cultures of the same age as a source of inoculum. A single paramecium was isolated into each of the depressions of a clear nonradioactive pyrex glass spot plate and the number of organisms living at the end of the experimental period was determined. At the time of starting the amount of food was adequate and roughly the same in each depression of both series. Each depression is considered as one culture. Experimental and control

TABLE I. Influence on Population of Radiation from Radioactive Sulfur, 13 Experiments.

Radiation /hr (rep)	Avg No. of paramecia/culture		No. of cultures (same in exp. and control)	P (<)
	Exp.	Control		
3.13	158.8	96.1	9	.01
3.13	154.0	91.5	9	.01
3.13	155	96	9	.01
3.13	36.6	9	36	.01
3.13	11.2	6.9	36	.02
3.13	11.0	3	36	.01
1.55	152.8	81.4	9	.01
1.55	151.4	83	9	.02
1.55	152	78	9	.01
.77	133	110	9	.02
.77	110	62	9	.05
.38	91	105	9	.4
.38	106	119.4	9	.6

cultures were kept in the same moist chamber. Room temperature varied about 5°C during the course of any one experiment. The experiments were done at various times over a period of several months. The radioactive sulfur was secured from Oak Ridge, in the form of labeled, carrier-free sulfuric acid in 0.1 N hydrochloric acid and had, when received, an activity of 12.6 millicuries per ml. Neutralization was done with NaOH and where necessary, because of high concentration, the Na content of experimental and control solutions was equalized. The concentration of sulfate as used for experimental solutions was so low (0.0005 mg/ml) that it was not considered necessary to equalize the amount in the 2 groups of cultures. The desired amount of radiation was secured by dilution, correction being made for the decay of sulfur activity. The radiation from S 35 is pure beta having an average energy of 0.055 Mev. There may be a small amount, of the order of a few microcuries per milliliter, of contaminating P 32 in the material as received from Oak Ridge. Calculation of the energy absorbed in the medium has been made using the S 35 beta energy and the measured number of disintegrations per second per milliliter of solution as used in the cultures.

The data from the experiments with S 35 are shown in Table I. The data show that the number of organisms developing in the sulfur solutions, containing between 1.5 and 3.1 rep per hour of beta activity, was significantly greater than in control solutions* It is not safe to compare the actual numbers of paramecia produced in experiments done at different times or to consider that controls are indiscriminately applicable. This is especially well shown in experiments 4, 5, and 6 (3.13 rep/hr) in which conditions were obviously much less favorable for growth and development than in the first experiment. The data also indicate a loss of stimulation at dose rates of about 0.77 rep and below.

The sulfur experiments indicate quite clearly that relatively low doses of radiation over an extended period of time favor the growth and division of paramecium. Calculation shows that the energy liberated in the solution containing the greatest concentration of sulfur is equal to approximately 3.13 rep per hour or for the entire experimental period, 6 days, a total of 450 rep.

The stimulating effect of a relatively small total dose of beta particles given at a very low dose rate could not be reproduced by an equivalent amount of soft X-rays (48 KV, 50 ma) given in one dose requiring about 3 minutes for delivery. Four hundred fifty roentgens of X-ray were given to culture media, water used for diluting media, and to the paramecia. Several combinations of irradiated and unirradiated fluid and cells were tried, using a total of about 200 paramecia.

---

* In interpreting these differences it is necessary to bear in mind that small or moderate differences in rate of growth may result in very large differences in number of animals. If no animals die, and if we denote the final number of X-irradiated animals by N_r, the final number of control animals by N_c, the initial number (the same for both groups) by N_o, the number of divisions for the irradiated animals by d_r, for control animals by d_c then

$$N_r = N_o \cdot 2^{d_r}$$
$$N_c = N_o \cdot 2^{d_c}$$
$$\text{Log}_2 \frac{N_r}{N_c} = d_r - d_c$$

Thus the logarithm (to base 2) of the ratio of the final number of animals in the treated and control groups is equal to the difference in the number of divisions. Whenever N_r and N_c differ significantly so will any function of them (d_r and d_c) differ significantly. The results are essentially the same whether the computation is performed on the total or separately for each culture.

TABLE II. Effect of 4 r or 8 r/Hr on Number of Paramecia Produced in 53 Hr.*

Exp. No.	4 r/hr		Control		8 r/hr
1.	53		34		54
	52		36		48
	59		37		53
	41		7		45
Total	205	$(P = <.05)$	114	$(P = <.05)$	200
2.	66		39		59
	49		37		65
	65		38		57
	70		44		59
	250	$(P = <.01)$	158	$(P = <.01)$	240
3.	13		9		13
	13		7		12
	13		8		14
	9		8		12
	48	$(P = <.01)$	32	$(P = <.01)$	51

* Four plates of 9 depressions each (36 isolation cultures) in each group of each experiment. Figures in body of table are number of paramecia per plate of cultures. Totals are for 4 plates.

In no instance could we distinguish a difference in population between experimental and control cultures.

Since the single dose of X-rays produced no increase in growth and division we decided to try a very low dose rate for a relatively long time. A 200 KV machine operating at 0.3 ma was used. By suitably adjusting the distribution of the cultures in the radiation field it was possible to secure dose rates of 4 and 8 r per hour and to irradiate both groups simultaneously. Controls were kept in the room in which the irradiations were made but were completely shielded by a lead box. Temperature of experimental and control cultures differed randomly by one or 2 degrees.

Three experiments with low X-ray dose rate were made. Each experiment contained 36 isolation cultures set up in 4 pyrex spot plates. All experiments covered an irradiation period of 53 hours. Results of these experiments are shown in Table II. There is a highly significant difference between the number of paramecia produced in the irradiated and control cultures. Apparently conditions in one control dish of Exp. 1 were not comparable with the other in the series. When this aberrant unit is included in the calculation $P = <.05$. If it is left out of consideration $P = <.01$.

In order to test whether or not the observed increased rate of growth and division of the irradiated cell was a permanent characteristic or a transient phenomenon the following experiment was made. Immediately after determining the number of cells in control and experimental cultures of Exp. 2, Table II, 18 paramecia were chosen at random from each of the experimental and control groups and set up in isolation cultures. No further irradiation was given to any of the paramecia. The total number of paramecia in the 3 groups at the end of 53 hours was: controls, 186; 4 r/hr, 220; 8 r/hr, 226. There is no significant difference between these groups.

In view of the results the likelihood of a mutation involving either nucleus or cytoplasm or both is indeed remote. Mutation cannot be operative in this case unless one assumes a fortuitous selection of a group of animals showing a rapidly developing forward mutation and an equally rapid and very precise back mutation.

The data show clearly that under certain ill-defined but reproducible conditions cells capable of autonomous existence are stimulated to grow and divide by continuous exposure to very low dose rates of either corpuscular or electromagnetic radiation of high energy. Experiments, carried out in the radioactive slides, where only a single individual was continuously exposed to the irradiation (Exp. 3) indicate strongly that the increase in population is not a response to alterations in the environment produced by products arising from injured cells of the same species as those used as experimental material. Biological entities can show functional stimulation under conditions such that no tissue injury can contribute to the increased activity associated with the absorption of high energy radiation by the reacting system. At the present time one can only speculate concerning the modes of action and sites of origin of the stimulant. An attractive and plausible hypothesis may be based on the production of free radicals by the absorbed energy and the stimulation by such radicals of metabolic activities. Low dose rates would produce radicals or other reaction products in amounts small enough to be stimulating while high dose rates would lead to toxic concentrations of the same or other substances.

Summary. Beta radiation administered at a level of from 0.77 to 3.13 rep per hour and X-ray radiation given at 4 or 8 r per hour stimulates reproduction in paramecium. The stimulation is not due to mutation since it disappears immediately on removal of the cell from the influence of the radiation.

Received May 21, 1953. P.S.E.B.M., 1953, v83.

Effect of Intravenous Administration of Paritol-C* on Serum Lipids of Hypercholesterolemic Rabbits.† (20451)

J. C. FORBES AND O. M. PETTERSON.

From the Department of Biochemistry, Medical College of Virginia, Richmond.

Previous work in our laboratory showed that only a relatively small percentage of the cholesterol of lyophilized normal human or rabbit serum can be extracted by cold chloroform even in 24 hours, while a large percentage of the serum cholesterol of nephrotic patients and hypercholesterolemic rabbits can be thus extracted(1). The fraction extractable in a 3-hour period, which is usually practically the same as extracted in 24 hours, has been called the "readily extractable" cholesterol.

Gofman and associates(2,3) have shown that heparin administration causes a shift in the lipoproteins of high Sf values into those of successively lower ones in the serum of hypercholesterolemic rabbits and human subjects. Nikkilä(4) obtained the same general effect. Injection of heparin also has been shown to suppress the rate of development of atherosclerosis in cholesterol fed rabbits(3). Since the anticoagulant Paritol exerts the same general effect as heparin on blood coagulation and has been shown to affect serum lipoproteins (5), it was decided to determine if its administration would favorably influence the concentration of the "readily extractable" fraction and that of total cholesterol in hypercholesterolemic rabbits.

Experimental. Male rabbits, weighing between 4 and 5 lb except when otherwise stated, were put on a 0.5% cholesterol diet prepared as follows. Fifty g of cholesterol were dissolved in 100 ml of chloroform and added to 500 g of Wesson oil. After warming on a water bath until practically all of the cholesterol was again in solution, the whole was added to 9450 g of Rockland rabbit pellets and thoroughly mixed. The diet was ready for feeding after all the chloroform had evaporated. It was fed to all the animals throughout the whole experimental period. The Paritol was injected intravenously using a 5% solution. Blood for the analytical studies was obtained by nicking an ear vein after the ear had been warmed with a heat lamp. The analytical procedures employed have been described previously(1).

In Exp. 1, after the animals were on the high cholesterol diet for about 12 weeks, the Paritol injections were begun. The total cholesterol by this time had reached an average of 1750 mg/100 ml of serum and the "readily extractable" fraction constituted 90% of the whole. The Paritol injections were given in amounts corresponding to 0.1 ml of a 5% solution/lb body weight. It will be seen from the results, which have been averaged in Table I, that the Paritol injections caused a marked drop in all the fractions studied. This was true in each individual animal as well as in the average for the group. The percentage of the total cholesterol present in the "readily extractable" form tended to decrease as the total cholesterol decreased. This was very marked in all individual cases where the total cholesterol had dropped to

---

* A polysulfuric acid ester of polyanhydromanuronic acid kindly supplied by Wyeth, Inc., Philadelphia.

† Presented at the annual meeting of the Virginia Academy of Science, May, 1953.

TABLE I. Effect of Intravenous Administration of Paritol-C on Serum Lipids of Hypercholesterolemic Rabbits.

		Avg conc. of serum lipids					
Exp. No.	No. of animals	T. chol.	R.E. chol.	N.F.+ chol.	R.E. chol. as % T. chol.	Serum collection, total elapsed time, days	Remarks
		%	%	%			High cholesterol diet 12 wk
1	8*	1.75	1.57	2.61	90	0	Control sera before Paritol
		1.09	.80	1.58	73	3	Same animals, Paritol 2 days. Bled 24 hr after last inj.
		.76	.54	1.36	71	6	Same animals, Paritol 2 additional inj. Bled 24 hr after last inj.
							High cholesterol diet 3 wk
2	4*	1.46	1.30	2.65	90	0	Control sera before Paritol
		.87	.58	1.31	67	14	Same animals, Paritol 2 inj. a wk for 2 wk. Bled 72 hr after last inj.
		.76	.64	1.31	84	28	Same after 3 inj. a wk for 2 more wk. Bled 48 hr after last inj.
		.74	.54	1.17	73	42	Same after similar inj. for 2 more wk. Bled 48 hr after last inj.
	3	.90	.75	1.32	83	0	Pair-fed controls. No Paritol
		1.31	1.08	2.00	82	14	Animals bled at same time as corresponding Paritol inj. animals
		1.76	1.67	2.88	95	28	
		2.69	2.48	4.33	92	42	
							High cholesterol diet 6 wk
3	3*	2.29	2.13	3.46	93	0	Control sera before Paritol
		1.18	1.14	2.00	97	5	Same animals, Paritol 2 days. Bled 72 hr after last inj.
		1.25	1.13	2.00	90	9	Same after 2 inj. alternate days. Bled 48 hr after last inj.
		.87	.62	1.30	71	14	Same after 2 more inj. alternate days. Bled 72 hr after last inj.
		.68	.31	1.10	45	21	Same after 3 more inj. alternate days, then 2 inj. consecutive days. Bled 24 hr after last inj.
		.69	.30	1.05	43	26	Same after 4 more inj. consecutive days. Bled 48 hr after last inj.
	2	2.27	2.12	3.39	93	0	Pair-fed controls. No Paritol.
		1.82	1.83	3.52	100	5	Animals bled at same time as corresponding Paritol inj. animals
		1.54	1.36	2.24	88	9	
		1.68	1.63	2.44	97	14	
		1.57	1.43	2.35	91	21	
		1.40	1.17	2.02	83	26	

* Blood samples from Paritol-injected animals were obtained just before next injection.

T. chol. = Total cholesterol. R.E. chol. = Readily extractable cholesterol. N.F. + chol. = Neutral fat plus cholesterol.

around 700 mg %. Actually no test animal in any experiment with a total cholesterol below 600 mg % showed over 40% of the total cholesterol present in the "readily extractable" form.

In Exp. 2 the animals were on the high cholesterol diet for only 3 weeks when the Paritol injections were begun. Four of the animals were given Paritol and 3 were kept as controls so as to show the effect of the diet alone on the lipid fractions under study. Each injected animal received 1 ml of the 5% Paritol solution at each injection. All animals were fed throughout the whole experimental period, except for the first couple of days, 20 g of diet/lb of body weight as determined at the time of the first Paritol injection. The injected animals ate very poorly for 2 days following the first Paritol injection, so the food intake of the controls was kept down to 10 g/lb of body weight during this period. Subsequent injections of the anticoagulant did not affect the animals' appetites in any way and all ate the full 20 g/lb of body weight, except on several occasions when a small amount was left, especially by the controls. It will be seen that the Paritol injections caused a drop of around 50% in the concentration of the various lipid fractions, while at the same time the concentrations of these fractions in the sera of the control animals rose to a high level during the same period. In the control series (Table I, Exp. 2) 2 of the animals were fairly resistant to the development of hypercholesterolemia as indicated by a considerably lower total cholesterol level at the time the Paritol injections were begun, but developed high values as the experiment progressed. All animals gained between 1.25 and 1.5 lb in weight during the experimental period. The animals used in Exp. 3 were in the laboratory for several months before they were used and were considerably larger than those used in the other experiments. Their general appetite was considerably less than that of the animals used in Exp. 2. They were on the cholesterol diet for 6 weeks before the injections were begun and by this time the average total cholesterol was 2280 mg % of which the "readily extractable" fraction constituted over 90%. Since the animals were not eating well, only 10 g of food/lb body weight was fed each day. The test animals always ate their food but one control often left some. This animal, however, in spite of its lower food consumption, consistently exhibited a higher serum cholesterol concentration than its accompanying control. The same general results were obtained in this experiment as in the previous ones. In this experiment, however, even the control animals showed a decided drop in the various fractions, but it was small compared with that of the injected animals. The difference in the response of the controls in this experiment and in Exp. 2 is undoubtedly due to the fact that their food consumption, and consequently cholesterol intake/lb of body weight was only about 50% of that of the animals in Exp. 2.

When the data are examined as a whole, it will be seen that the changes in the neutral fat plus cholesterol tended to follow rather closely those of total cholesterol, a decrease or increase in one being usually accompanied by a roughly proportional change in the other. Since the neutral fat plus cholesterol fraction includes free cholesterol, ester cholesterol, and neutral fat the changes noted in this fraction are additional evidence in support of the observed changes in total cholesterol following the Paritol injections.

Phospholipid phosphorus determinations were carried out on all sera and they were found to vary more or less with the total cholesterol. The anticoagulant Dicumarol was injected intravenously at a level of 50 mg/day for 2 days into several animals without any effect on any of the lipid fractions being studied.

Summary. The intravenous injection of Paritol-C into hypercholesterolemic rabbits has been shown to cause a marked drop in the "readily extractable" cholesterol fraction, as well as in total cholesterol.

---

1. Forbes, J. C., Dillard, G. H. L., Porter, W. B., and Petterson, O., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 240.

2. Lyon, T. P., Jones, H. B., Graham, D. M., Gofman, J. W., Lingren, F. T., and Yankley, A., *Arch. Int. Med.*, 1952, v89, 421.

3. Graham, D. M., Lyons, T. P., Gofman, J. W., Jones, H. B., Yankley, A., Simonton, J., and White, S., *Circulation*, 1951, v4, 666.

4. Nikkilä, E. A., *Scand. J. Clin. and Lab. Invest.*, 1952, v4, 369.

5. Hoch, H., and Chanutin, A., *J. Biol. Chem.*, 1952, v197, 503.

Received June 1, 1953. P.S.E.B.M., 1953, v83.

Participation of Erythromycin and Carbomycin in Combined Antibiotic Action *in vitro*.* (20452)

V. R. Coleman, J. B. Gunnison, and E. Jawetz.

From the Department of Microbiology, University of California School of Medicine, San Francisco.

Recently two new antimicrobial agents, erythromycin and carbomycin, have appeared on the market(1,2). Early work on these drugs has indicated that their antimicrobial spectrum is very similar(3), that they give nearly complete cross-resistance(4), and that drug-resistant variants emerge quite rapidly from bacterial populations(3). It has been pointed out that simultaneous exposure of bacterial populations to erythromycin and either penicillin or streptomycin greatly diminishes the frequency of such resistant variants(5). For this reason it appeared likely that these new drugs might often be used in combinations with other antimicrobial agents. It was of interest, therefore, to examine the behavior of erythromycin and carbomycin when combined with other drugs, particularly with regard to the occurrence of synergism or antagonism.

Earlier reports from this laboratory have dealt with combined antibiotic action as manifested by the rate of bactericidal action(6-8). From the standpoint of bactericidal rate *in vitro* and *in vivo* the following scheme of combined antibiotic action has been proposed(9): Antibiotics fall into 2 broad groups: I. penicillin, streptomycin, bacitracin, neomycin. II. aureomycin, chloramphenicol, terramycin. Members of Group I are often synergistic with one another, sometimes indifferent, never antagonistic. Members of Group II are neither synergistic nor antagonistic with one another but may show simple additive effects. A combination of Group I with a Group II antibiotic may result in either indifference, synergism, or antagonism, depending in part on the manifest behavior of the microorganism toward the individual drugs. This scheme has proved useful in planning experiments and may find application in combined antibiotic therapy in clinical medicine. Hence it seemed advisable to establish the position of the new antibiotics in this tentative scheme.

Materials and methods. The test organisms included *Klebsiella pneumoniae* (A-D), *Micrococcus pyogenes var. aureus* (Heatley), and *Streptococcus fecalis* (No. 16), all of which had been used in establishing the original division of the antibiotics into 2 groups(7). In addition, 3 strains of *M. pyogenes var. aureus* (Co, Da, and Sh) recently isolated from patients were tested. Commercial preparations of crystalline sodium penicillin G, streptomycin sulfate, and bacitracin were used. Terramycin hydrochloride was obtained from Dr. G. L. Hobby, chloramphenicol from Dr. G. Rieveschl, erythromycin from Dr. L. E. Josselyn, and carbomycin from Dr. Frederick C. Fink. The drugs were dissolved in 0.85% sodium chloride and final dilutions were made in broth immediately before use. Proteose No. 3 broth containing the diluted antibiotics was inoculated with an 18-hour broth culture of the test bacteria to give a concentration of 10^7 to 10^8 organisms per ml in a total volume of 15 ml. Samples of 0.5 ml were removed at intervals during incubation at 37°C and the number of viable bacteria was estimated by plate counts on Proteose No. 3 agar (Difco). Details of the method and its interpretation have been described(10).

Results. When acting alone, erythromycin and carbomycin behaved in a similar manner except that a larger dose of the latter was often required for an antibacterial effect. Both drugs in adequate doses exerted a bactericidal effect during the first 24 hours of action but some bacteria usually survived and subsequently multiplied.

When bacteriostatic or slowly bactericidal doses of either erythromycin or carbomycin were combined with a rapidly bactericidal dose of penicillin, these new drugs sometimes

---

* Supported in part by grants from the National Institutes of Health, U. S. Public Health Service, and the Research Committee of the University of California School of Medicine.

TABLE I. *In Vitro* Action of Erythromycin and Carbomycin with Penicillin, Streptomycin, or Bacitracin, Estimated by Plate Counts of Viable Bacteria.

Test organism	Drug and amt (μg/ml) (bacitracin in units/ml)	No. of viable bacteria/ml, Hr after inoculation: 0	7	24	32-48	Combined effect
Micrococcus pyogenes var. aureus (Heatley)	None	6×10^6	8×10^7	6×10^8	10^9	
	Penicillin .3		2×10^4	10^1	10^1	
	Erythromycin 2.0		2×10^6	8×10^4	6×10^4	
	Pen .3 + erythro 2.0		2×10^6	3×10^4	5×10^3	Antagonism
	Carbomycin 2.0		3×10^5	8×10^4	5×10^4	
	Pen .3 + carbo 2.0		5×10^5	2×10^4	5×10^3	"
Klebsiella pneumoniae A-D	None	10^7	4×10^8	6×10^7	10^8	
	Penicillin 6.0		4×10^4	10^1	3×10^1	
	Erythromycin 10.0		6×10^5	3×10^6	10^8	
	Pen 6.0 + erythro 10.0		5×10^5	2×10^3	8×10^2	Antagonism
	Streptomycin 5.0		3×10^3	3×10^7	10^8	
	Strepto 5.0 + erythro 10.0		4×10^2	10^1	10^1	Synergism*
Micrococcus pyogenes var. aureus (Co)	None	3×10^7	2×10^8	5×10^8	10^9	
	Bacitracin 1.0		6×10^7	5×10^8	8×10^8	
	Carbomycin 1.0		9×10^6	5×10^6	7×10^7	
	Baci 1.0 + carbo 1.0		5×10^5	2×10^2	10^2	Synergism*
Micrococcus pyogenes var. aureus (Sh)	None	2×10^7	3×10^8	6×10^8	10^9	
	Bacitracin 10.0		3×10^6	4×10^4	4×10^3	
	Erythromycin .2		3×10^7	4×10^7	7×10^7	
	Baci 5.0 + erythro .2		5×10^5	10^2	4×10^1	Synergism*

* Synergism denotes a bactericidal effect *greater* than that obtained with 2-5 times larger concentrations of each single drug, used alone.

TABLE II. Combined *In Vitro* Action of Erythromycin and Carbomycin with Penicillin or Bacitracin.

Antibiotic combination	Test organisms: *M. pyogenes var. aureus* strain H	Da	Sh	Co	*K. pneumoniae* strain A-D	*S. fecalis* strain #16
P + T	Antag.	Indiff.	Indiff.		Antag.	Antag.
P + E	"	"	"		"	"
P + C	"	"	"		"	"
S + E				Indiff.	Synerg.	
S + C				Addit.	Addit.	
B + T	Synerg.	Addit.	Synerg.	"		
B + E	Indiff.	"	"	"		
B + C	"	"		Synerg.		

Indifference = effect equal to that of the more active drug alone. Antagonism = effect less than that of the more active drug alone. Addition = simple algebraic summation. Synergism = effect greater than simple algebraic summation of single drug effects. Blank = not tested.

P = penicillin; B = bacitracin; E = erythromycin; C = carbomycin; T = terramycin; S = streptomycin.

reduced the bactericidal rate of the penicillin. Both erythromycin and carbomycin interfered with the bactericidal effect of penicillin acting on the test strains of *K. pneumoniae, S. fecalis,* and *M. pyogenes var. aureus* (Heatley) (Tables I and II). This antagonism was similar to that previously shown for chloramphenicol, aureomycin, and terramycin using the same test organisms(8,10,11). On the other hand, against *M. pyogenes var. aureus* (Sh and Co), erythromycin was synergistic with the Group I drug, bacitracin. Combinations of bacteriostatic doses of the new drugs with bacteriostatic doses of Group I drugs often showed an additive effect. A similar relationship has been established for bacteriostatic amounts of Group I with Group II drugs(8). Hence, erythromycin and carbomycin behaved in a manner resembling Group II antibiotics (Table II).

Combinations of erythromycin with carbomycin had only negligible additive effects. Mixtures of either chloramphenicol or terramycin with these new agents resulted in a combined effect no greater than that obtained by increasing proportionately the dose of either drug used singly. In this regard also erythromycin and carbomycin behaved like members of Group II.

Antibiotics of Group II are, to some extent, interchangeable in combinations. Erythromycin or carbomycin, however, could not always replace terramycin or aureomycin. For example, when tested against *M. pyogenes var. aureus* (H) bacitracin and terramycin were synergistic(7) but neither erythromycin nor carbomycin showed this effect when combined with bacitracin. Using another strain of *M. pyogenes var. aureus* (Sh), it was shown that erythromycin and bacitracin were synergistic over a wide range of concentrations including bactericidal doses of bacitracin. Against a third strain of this organism (Da), bacteriostatic doses of bacitracin were additive when combined with the new drugs (Table I). As with other Group II antibiotics, the behavior of erythromycin and carbomycin in combination with Group I drugs was unpredictable and seemed to depend, at least in part, upon the sensitivity of the test bacteria to the Group I drug. The available evidence therefore placed erythromycin and carbomycin into Group II of the above scheme. A paper by Rantz and Randall(12) published while this work was in progress likewise places erythromycin into Group II. Experiments are in progress to confirm this *in vitro* classification by results of treatment of experimental infections.

There are two principal reasons for the clinical simultaneous use of 2 antimicrobial drugs in treating an infection due to a single microbial species(6,13): a) One antimicrobial drug delays the emergence of variants resistant to the other drug. b) The bactericidal rate or bacteriostatic effect of the drug mixture is far greater than that of either drug alone in comparable concentration. It had already been shown that the addition of Group I drugs to erythromycin or carbomycin delays the emergence of resistant variants which tend to shorten greatly the useful length of drug administration(5). The work presented here classifies erythromycin and carbomycin with other Group II agents and indicates that on occasions striking synergism with Group I drugs may be encountered. Using a survey technic to be reported later, both erythromycin and carbomycin showed some form of positive summation of action in combination with Group I drugs against 11 strains of recently isolated *M. pyogenes*. The antagonism between these agents and Group I antibiotics, as with other Group II drugs, is probably of limited clinical significance for reasons described elsewhere(6). Therefore, it appears that use of erythromycin and carbomycin in combination with other antimicrobial agents deserves consideration.

Summary. With respect to their behavior in combinations with other antibiotics, as measured by bactericidal rate *in vitro*, erythromycin and carbomycin fall in a group with aureomycin, chloramphenicol and terramycin. The classification of these agents in a tentative scheme of combined antibiotic action is discussed in terms of laboratory tests and clinical application.

---

1. McGuire, J. M., Bunch, R. L., Anderson, R. C., Boaz, H. E., Flynn, E. H., Powell, H. M., and Smith, J. W., *Antibiot. and Chemother.*, 1952, v2, 281.

2. Tanner, F. W., Jr., English, A. R., Lees, T. M., and Routien, J. B., *Antibiot. and Chemother.*, 1952, v2, 441.

3. Haight, T. H., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 175.

4. Finland, M., Wilcox, C., Wright, S. S., and Purcell, E. M., PROC. SOC. EXP. BIOL. AND MED., 1953, v81, 725.

5. Purcell, E. M., Wright, S. S., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 124.

6. Jawetz, E., *Arch. Int. Med.*, 1952, v90, 301.

7. Jawetz, E., Gunnison, J. B., Bruff, J. A., and Coleman, V. R., *J. Bact.*, 1952, v64, 29.

8. Gunnison, J. B., Shevky, M. C., Bruff, J. A., Coleman, V. R., and Jawetz, E., *J. Bact.*, in press.

9. Jawetz, E., and Gunnison, J. B., *Antibiot. and Chemother.*, 1952, v2, 243.

10. Gunnison, J. B., Jawetz, E., and Coleman, V. R., *J. Lab. and Clin. Med.*, 1950, v36, 900.

11. Gunnison, J. B., Coleman, V. R., and Jawetz, E., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 549.

12. Rantz, L. A., and Randall, E., *Antibiot. and Chemother.*, 1950, v2, 645.

13. Dowling, H. F., Lepper, M. H., and Jackson, G. G., *J.A.M.A.*, 1953, v151, 813.

Received June 2, 1953. P.S.E.B.M., 1953, v83.

An Antineoplastic Factor in Spleens of Mice Previously Inoculated with Methylcholanthrene. (20453)

Morris Pollard and Robert H. Bussell.
From the University of Texas Medical Branch, Galveston.

When carcinogenic compounds are applied to, inoculated into, or fed to rodents, the host may eventually develop a tumor either at the site of the exposure or, selectively, in such organ as the liver. The wide variety of such compounds has been well catalogued by Hartwell(1), but the mechanism of this response has not been clearly defined. Some compounds induce tumors more rapidly than others (*e.g.* methylcholanthrene vs. 1,2,5,6-dibenzanthracene), but all require relatively prolonged exposure to the host's tissues before a neoplasm appears.

It was observed in this laboratory(2) that when a carcinogenically-induced transmissible tumor involuted, the recovered host was refractory to reinoculation with the same tumor. This "immunity" was recorded by Jensen(3), Ehrlich(4), and more recently by Mazwell and Aptekan(5). In our study the "immune" phenomenon was associated with tissue cultures of growing splenic tissue. Using a similar tissue culture technic, involving two types of tissue per tube, the response of the mouse to methylcholanthrene inoculation was studied. Tumor and spleen tissues were grown simultaneously in the same tube, and observations were made on the effect of the spleen tissue on tumor tissue.

Methods. All of the C3H mice used in this study were purchased from the Jackson Memorial Laboratory, Bar Harbor, Maine. Groups of mice were inoculated subcutaneously with 0.5 mg of 20-methylcholanthrene* in cooking oil. Fibrosarcomas appeared at the point of inoculation by the 45th day and such tumors continued to appear in the rest during the 2 months following. The tumors and spleens from these mice were used for the study described below. Groups of 15 mice were each inoculated subcutaneously with 0.5 mg of 20-methylcholanthrene,* 0.5 mg of 3,4-benzpyrene,* 0.5 mg of 1,2,5,6-dibenzanthracene,* and with phenanthrene. All of these compounds were suspended in cooking oil. Cooking oil alone was also inoculated into a group of mice. At weekly intervals thereafter, 3-4 mice from each group were sacrificed and their spleens were studied for their destructive capacity on the methylcholanthrene-induced tumors referred to above. Each experiment was repeated at least 3 times, and the 2-week studies were repeated 7 times. The tumor and spleen tissues were individually minced with scissors and the fragments (ca. 1.0 mm in diameter) were washed 3 times in Hank's solution. Three or 4 pieces of tumor were explanted in plasma on one wall of a tube and to the opposite side about 3-4 times as many spleen particles were explanted. Each minced spleen was divided among 3-4 tubes. After the addition of one ml nutrient fluid,† the tubes were stoppered and rotated 6 times per hour at 35°C. Tubes with tumor alone, spleen alone, and tumor plus "normal" spleen were also simultaneously studied. In evaluating the significance of the various combinations of tissues, it was felt that in all instances the spleen tissues must show evidence of growth in order that the growth characteristics of the tumor tissue be significant. Furthermore, the tumor and spleen tissues alone must show good growth properties.

The magnified explants were photographed‡ through the wall of the tube thus obviating the need for disturbing the tissues. For simplicity, the spleen tissues are referred to as 1) homologous—from the mouse supplying the tumor, 2) methylcholanthrene—indicating the weeks from inoculation, 3) phenanthrene—indicating the weeks from inoculation, and 4) "normal"—from a mouse that had not been inoculated.

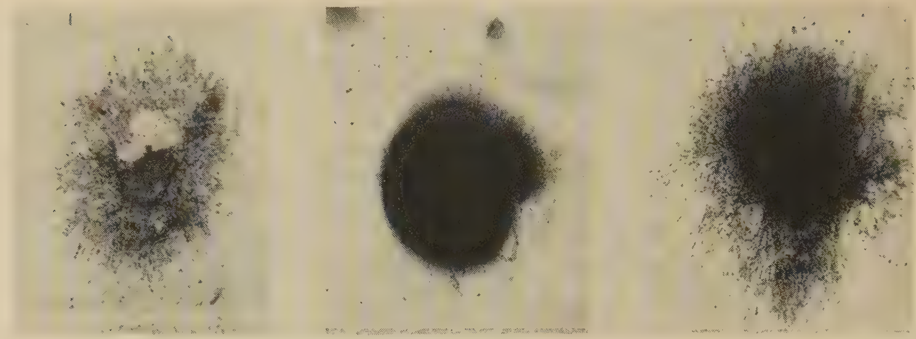
Results. Tumor explants grew profusely

---

* Eastman Kodak Co.

† Ox serum ultrafiltrate, Hank's solution, and chick embryo extract.

‡ We gratefully acknowledge the kind assistance of Mr. F. W. Schmidt, Dept. of Medical Photography, University of Texas Medical Branch.

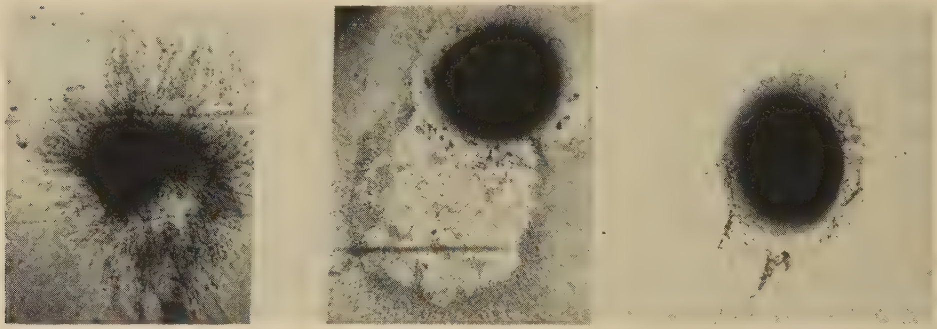


Tumor alone

With spleen of methylcholanthrene-inoculated mouse (1 wk previously)

With homologous spleen

FIG. 1. Tissue cultures of methylcholanthrene-induced mouse tumor—four days. ×38.

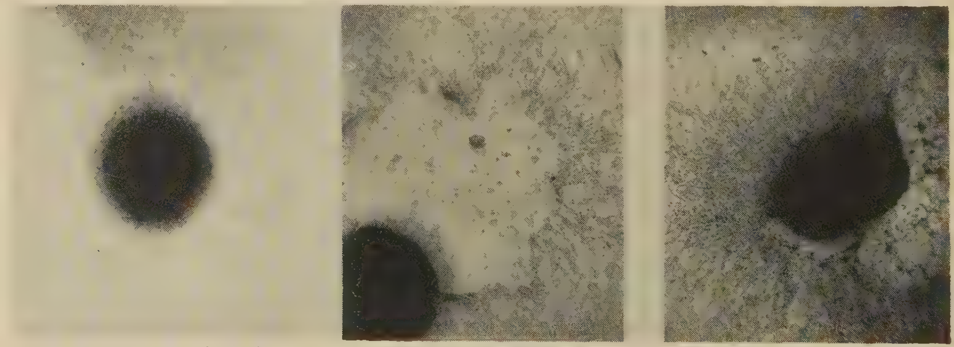


+ normal C_3H spleen

+ C_3H phenanthrene spleen (2 wk)

+ C_3H methylcholanthrene spleen (2 wk)

FIG. 2. Tissue cultures of methylcholanthrene-induced mouse tumor—two days. ×36.



+ meth. spleen (2 wk)

+ meth. spleen (3 wk)

+ meth. spleen (4 wk)

FIG. 3. Tissue cultures of methylcholanthrene-induced mouse tumor. ×30.

alone, and in the presence of homologous and "normal" spleen tissues (Fig. 1). Within 24 hours, fibroblasts grew out radially from the explant, with occasional areas of liquefaction within the growing "halo" of cells. Twelve tumors were grown with their homologous spleens and in no instance was any tumor suppressive effect detected.

Tumor explants grew poorly and most of them died when explanted in the same tube with spleen tissues from mice inoculated with methylcholanthrene one and 2 weeks previously (Fig. 1 and 2). This reaction was demonstrable in 6 of 8 experiments. The antineoplastic (AN) effect was less marked at 3 weeks after inoculation and thereafter the tumor growth resembled that which grew with homologous spleen (Fig. 3). The antineoplastic effect appeared on the first day after explantation and appeared complete during the next 2 days. Under these conditions, most of the tumor explants failed to grow, or grew sparsely, were disintegrated and dead within the next 2 days. The mice supplying the spleens at 1-4 weeks after inoculation showed local evidence of the oily inoculum but no visible trace of neoplasm.

Tumor explants grew profusely in the same tube with phenanthrene spleens (Fig. 2), as well as with spleens from mice previously inoculated with benzpyrene and with 1,2,5,6-dibenzanthracene. Their growth pattern did not differ from those growing with "normal" and with homologous spleens. The growth of splenic tissue explants followed a pattern of cell migration within 24 hours of explantation. Within the next 2 days, fibrocytes and large lymphoid cells appeared at the periphery of the explant. Those spleen explants which exerted a tumor antagonistic effect were surrounded by the lymphoid cells, whereas the others exhibited a fibroblastic outgrowth.

Discussion. The A N phenomenon described above has not yet been associated with a soluble antibody; however, we shall refer to it as an "immune" reaction until evidence warrants a change. This transient phenomenon has manifested some degree of chemical specificity: phenanthrene and cooking oil injections alone did not induce the A N effect on methylcholanthrene-induced tumors, nor did 3,4-benzpyrene and 1,2,5,6-dibenzanthracene. The A N reaction was not uniform with all mice receiving methylcholanthrene. Absence of the A N reaction might be attributable to 1) an inhomogeneity among the animals used, 2) multiple influences of methylcholanthrene, 3) a delayed A N response, or 4) an A N period which disappears before one week. Exploratory tests for the latter proposal have indicated that the A N factor does not appear in the spleens of inoculated animals in less than 6 days.

The 3-week period of A N response might coincide with the latent period of carcinogenesis in the host. Since carcinogenic agents vary in this property, some of the slower acting agents should be studied with the technic described above, to see if the A N period is prolonged.

The mechanism of the A N reaction may simulate the "immunological paralysis" described by Felton and Ottinger(6). When they inoculated massive doses of pneumococcus polysaccharide into mice, the immunogenic mechanism failed. Strong's statement(7) that "all carcinogens are mutagens" may be pertinent to this A N process: if a somatic mutation induces a new antigenic specificity in tissues, then the antigenic burden thus imposed through methylcholanthrene might eventually cause the protective mechanism of the host to fail. This may explain the observed response of the host to methylcholanthrene, in which the host's spleen tissues manifested chronologically an initial destructive effect then a tolerant effect on tumor tissue to which it was exposed.

Summary. C3H mice were inoculated subcutaneously with 0.5 mg of 20-methylcholanthrene. At weekly intervals thereafter, spleens were examined from groups of mice for growth effect on methylcholanthrene-induced tumors. By growing spleen and tumor explants in the same tube, a tumor-destructive effect could be demonstrated in spleens for as long as 3 weeks after inoculation. Thereafter, this effect could not be detected. The tumor-destructive effect could not be demonstrated with "normal" spleens, nor with the spleens from the mice supplying the tumor tissue. This reaction could not be induced with phenanthrene, 3,4-benzpyrene, 1,2,5,6-dibenzanthracene, and the cooking oil in which these compounds were dissolved.

---

1. Hartwell, J. L., Survey of compounds which have been tested for carcinogenic activity, *U. S. P. H. S. Pub. No.* 149, 1951, pp 1-583.

2. Pollard, M., and Bussell, R., *Tex. Rep. Biol. and Med.*, 1953, v11, 48.

3. Jensen, C. O., *Hospitalstid*, 1903, v11, 549.

4. Ehrlich, P., *Arb. aus dem Konig. Inst. f. Exp. Therap. zu Frankfurt A. M.*, Verlag Gustav Fischer, 1906, 77-102.

5. Mazwell, L. D., and Aptekman, P., *Surgery*, 1951, v30, 689.

6. Felton, L. D., and Ottinger, B., *J. Bact.*, 1942, v43, 94.

7. Strong, L. C., *Brit. J. Cancer*, 1949, v3, 97.

Received June 8, 1953. P.S.E.B.M., 1953, v83.

Influence of Nitrogen Intake on Nitrogen-Retaining Action of Testosterone Propionate. (20454)

PRESTON L. PERLMAN AND JAMES W. CASSIDY.
From the Biological Research Laboratories, Schering Corp., Bloomfield, N. J.

With the discovery(1) that androgens induce nitrogen retention in the castrate dog, much effort has been expended in elucidating the nature of the response and the factors regulating it. Our interest in the problem was stimulated by the fact that only fragmentary information was available regarding the influence of nutritional status and, particularly, nitrogen plane on the degree of response to testosterone. Kochakian and Van Der Mark (2) have recently confirmed and extended their earlier findings that "once an adequate protein intake is provided a further increase in the protein (intake) of the rat . . . does not enhance the overall protein anabolic activity of testosterone." Earlier, Albright and his associates(3) expressed the view that the absolute influence of androgen on urinary nitrogen should diminish as the protein consumption declines, and Kenyon and Knowlton(4) had indeed demonstrated such a tendency in a study on the normal, human adult.

It appeared important, therefore, to determine the response when the level of nitrogen intake was reduced to the minimal maintenance requirement. At this low level of nitrogen intake, it should also be possible to determine whether or not testosterone functions by "facilitating", *i.e.*, making more efficient, the utilization of nitrogen, an hypothesis that we have considered as one possible explanation of the mechanism of action.

Methods. The 2 dogs used were pedigreed, adult, male beagles that had been castrated approximately one year prior to use in this study. Each dog was housed in an individual metabolism cage throughout the entire investigation. The animals were fed daily at the same time; the composition of the iso-caloric, high nitrogen and low nitrogen diets are given in Table I. The nitrogen contents of the diets were determined at frequent intervals by Kjeldahl analyses. Urines were collected under toluene and the nitrogen content determined daily using a micro-Kjeldahl distillation procedure. Fecal nitrogen was not done routinely but was determined at frequent times during the course of the study. The results were sufficiently close to 10% of the dietary intake to justify the use of this figure for computing the fecal N contribution to the daily total N excretion. The average N excretion (urine plus calculated fecal) for 3 consecutive days constituted one collection period. Testosterone propionate (25 mg/ml in sesame oil) was injected, intramuscularly, for the periods indicated in Fig. 1. The low nitrogen diet (0.20 g N/kg b.wt.) was selected experimentally and was at a level for Dog VII, which would just maintain this animal in nitrogen equilibrium. Below this intake positive nitrogen could not be obtained. For Dog V, this N-level produced a slightly negative balance.

TABLE I. Composition of Diets* (g %).

	A	B
Casein	23.5	11.8
Sucrose	53.0	65.0
Lard	18.5	18.2
Wesson's salts	2.0	2.0
Agar	3.0	3.0
Cal/kg b.wt provided	70	70
g N/kg b.wt provided	0.45	0.2

* Each animal received one high potency multivitamin capsule per day.

Results and discussion. The results of these experiments are presented in Fig. 1. It will be noted that both animals exhibited a significant nitrogen retention following the first administration (interval II) of testosterone propionate. The response persisted into the post-injection interval (interval III) which is in confirmation of the observations of Thorn and Engel(5). It is of interest that no significant nitrogen "rebound" was observed in any of the trials. When the animals were again subjected to testosterone propionate treatment (interval V), Dog VII exhibited a clear-cut nitrogen retention but the response of Dog V was not manifested until the

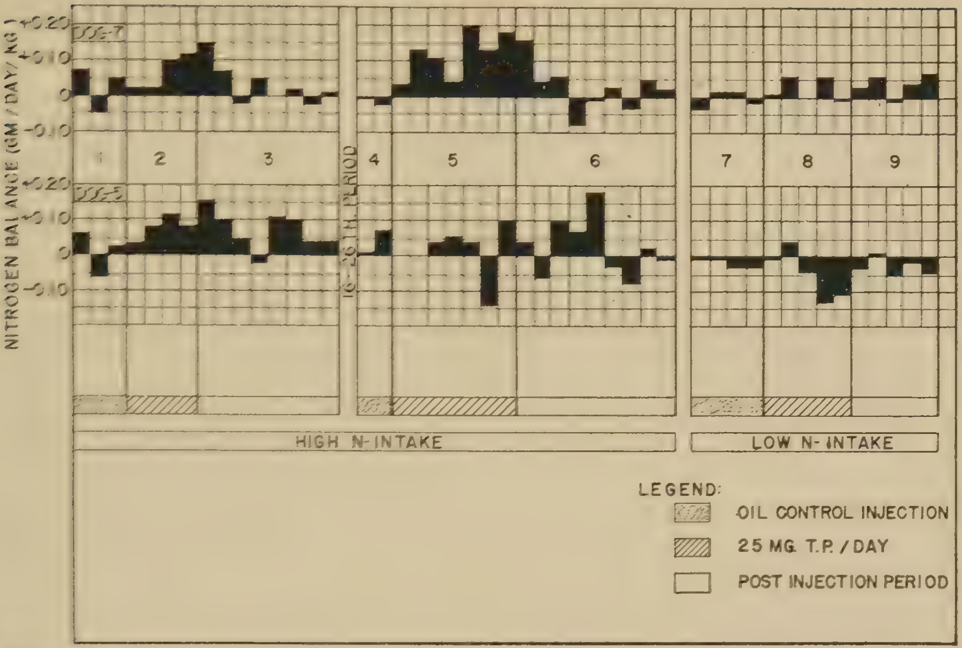


FIG. 1. Nitrogen retaining activity of testosterone propionate at two different levels of nitrogen intake in castrate dogs.

post-injection interval (interval VI).

The animals were next equilibrated on a low nitrogen intake (0.20 g N/kg/day) and when stabilized on the diet, testosterone propionate treatment was begun (interval VIII). No significant nitrogen retention occurred. In fact, it would appear that in Dog V, a more negative balance was obtained. The significance of this response is not clear but in certain experiments (unpublished) in this laboratory with rats on a minimal nitrogen intake, the occurrence of a strongly-negative nitrogen balance during treatment with testosterone propionate was also observed.

These data clearly demonstrate that in the region of the minimal, nitrogen-maintenance requirement, the nitrogen-retaining activity of testosterone propionate is absent. These results also make it improbable that testosterone acts by "facilitating" the utilization of dietary nitrogen. That the data presented may have certain clinical or therapeutic implications would appear obvious particularly when it is realized that individuals with very low nitrogen intakes may be in perfect balance. Hence, the mere presence of nitrogen equilibrium is not adequate in itself to assure a response to testosterone. A knowledge of the protein or other stores may be of more importance in determining whether or not a nitrogen retention can result from testosterone treatment.

Summary. The nitrogen-retaining activity of testosterone propionate in the castrate, male dog is manifested when the animal is maintained at levels above the minimal-maintenance nitrogen level of intake. At the minimal level of nitrogen, no nitrogen retention could be induced.

---

1. Kochakian, C. D., and Murlin, J. R., *J. Nutrition,* 1935, v10, 437.

2. Kochakian, C. D., and Van Der Mark, W., Proc. Soc. Exp. Biol. and Med., 1952, v79, 74.

3. Albright, F., *Macy Conf. on Bone and Wound Healing,* 1942, v1, 9.

4. Kenyon, A. T., and Knowlton, K., *Macy Conf. Metabol. Aspects Convalescence,* 1943, v4, 116.

5. Thorn, C. W., and Engel, L. L., *J. Exp. Med.,* 1938, v68, 299.

Received June 9, 1953. P.S.E.B.M., 1953, v83.

Egg Cultivated Tumor Protects Embryo against Vaccinia Virus. (20455)

Alfred Taylor and Nell Carmichael.

From the Biochemical Institute, University of Texas, and the Clayton Foundation for Research, Austin, Texas.

It has been demonstrated that some viruses possess oncolytic activity against certain tumors(1-3). The basis for these viral antitumor effects is still unknown. In some instances, it appears to be a direct effect analogous to that which occurs in nontumor cells when a virus manifests its usual pathogenic effects. It is also possible that if the tumor process is the result of the reaction of a cell to a specific tumor agent, some of the effects observed may be due to the interference of one virus with the action of another.

The work done in the past on this problem has been concerned with how a particular virus affects a tumor. The present investigation considers the effect of a growing tumor on the toxicity of a virus to the host organism.

Experimental. Embryonated eggs bearing tumors implanted by the yolk sac method and viruses adapted to egg cultivation were used in these studies. Tests were made with a C_3H mouse mammary adenocarcinoma, dba mouse mammary adenocarcinoma and a rat sarcoma. These tumors have been described elsewhere(4). Vaccinia was the principal virus utilized in the experiments, but tests were also made with Lee strain influenza. Seed viruses for the strains were obtained originally from Dr. McLean of Parke, Davis & Co. The eggs were implanted with tumor tissue on about the 4th day of incubation(5). The virus was introduced into the yolk sac on the 9th or 10th day, at which time the tumor tissue was well established but very small (0.02 g). At the conclusion of the tests the yolk sac tumors averaged 0.6 to 1.2 g. Vaccinia and influenza were used at concentrations which caused the death of the embryo in 4 and 3 days, respectively, in nontumor-bearing eggs. The tumor-bearing eggs were divided into 2 groups, one of which received injections of H_2O into the yolk sac, while the other group was injected in the same way with the virus suspension. A third group, which consisted of nontumor-bearing eggs at the same stage of incubation, were injected with the virus suspension. The nontumor-bearing virus inoculated eggs served as the control for virus toxicity, and the H_2O injected tumor-bearing eggs served as the control for embryo mortality due to tumor growth. In the work with vaccinia, 3 experiments involving 249 eggs were completed with the C_3H mammary tumor, 2 experiments including 113 eggs with the rat sarcoma, and 4 experiments utilizing 253 eggs with the dba mammary tumor. The same tumors were used in experiments with influenza but since the results were negative, no further details are necessary.

Results and discussion. The results of the tests with vaccinia and the various tumors are contained in Table I and Fig. 1, 2, and 3. It will be noted that embryos supporting implants of the dba mammary tumors (Table I and Fig. 1) were protected against the usual toxic effect of the virus. Tumor growth was not appreciably affected.

Allantoic fluid of the embryos of the tumor-bearing eggs which survived vaccinia inoculation contained live virus. Extracts made from these eggs had the same virus concentration as those made up from the nontumor eggs which died in the usual 3-4 day period.

It will be observed in Table I and Fig. 2 that the C_3H mammary tumor gave only a slight extension to the life of the embryo, while the rat sarcoma (Table I and Fig. 3) was ineffective. The C_3H tumor grew more rapidly than the dba and for that reason was more toxic to the embryo. It may be that this accounts for the difference in the action of the 2 mammary tumors. The rat sarcoma, however, grew more slowly than either of the other two and was particularly nontoxic, so that many of the embryos of eggs inoculated with this tumor survived to hatching time.

The yolk sac implanted tumor is so situated that embryo and tumor grow together sharing a common blood stream but otherwise not in-

TABLE I. Toxicity of Vaccinia to Embryos of Eggs Bearing dba Mouse Mammary Tumors, C_3H Mouse Mammary Tumors, and Rat Sarcomas.

	No. experiments	No. eggs	Mortality (days after virus inj.)													
			1		2		3		4		5		6		7	
			No. eggs dead	% dead	No. eggs dead	% dead	No. eggs dead	% dead	No. eggs dead	% dead	No. eggs dead	% dead	No. eggs dead	% dead	No. eggs dead	% dead
dba tumor control	4	85	20	24	23	27	29	34	37	44	44	52	55	65	73	86
dba tumor + virus	4	84	30	36	34	40	40	48	50	60	56	67	63	75	75	89
Nontumor + virus	4	84	14	17	16	19	33	39	79	94	84	100				
C_3H tumor control	3	95	18	19	30	32	36	38	45	47	51	54				
C_3H tumor + virus	3	95	12	13	18	19	48	51	84	88	95	100				
Nontumor + virus	3	59	10	17	19	32	48	81	59	100						
Rat tumor control	2	37	6	16	7	19	9	24	11	30	12	32				
Rat tumor + virus	2	38	7	18	16	42	32	84	37	97	38	100				
Nontumor + virus	2	38	6	16	9	24	34	90	38	100						

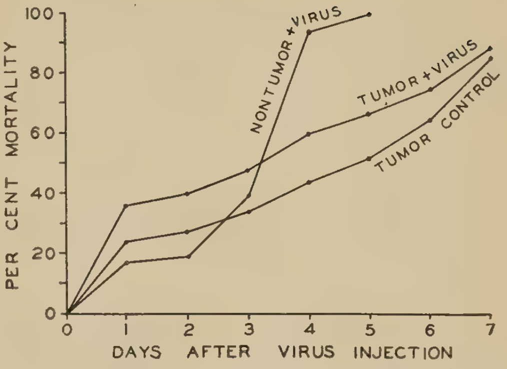


FIG. 1. Toxicity of vaccinia to embryos of eggs bearing dba mouse mammary tumors.

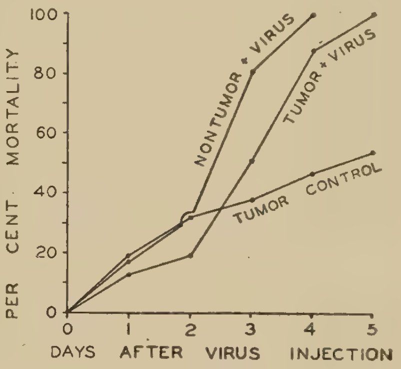


FIG. 2. Toxicity of vaccinia to embryos of eggs bearing C_3H mouse mammary tumors.

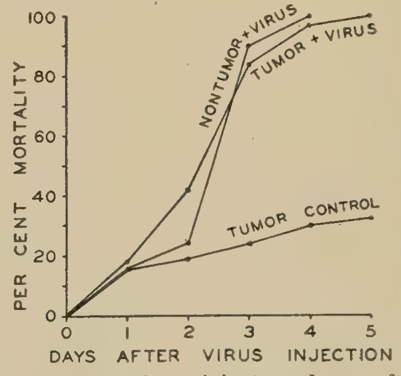


FIG. 3. Toxicity of vaccinia to embryos of eggs bearing rat sarcomas.

terfering with each other. The protection afforded the embryo by the dba tumor must have been due to something passed into the blood from the tumor tissue. Since the healthy, vigorous embryos continued to contain live virus in their fluids, it would appear that there was a constant neutralization of the virus toxicity.

Previous research with the dba mammary tumor used in this study has provided both direct and indirect evidence for the tumor agent or virus concept of tumor etiology(6-9). It is suggested that the present results contribute to the same hypothesis. The protection afforded the embryo against vaccinia by the implanted tumor could have been due to the well-known blocking effect which one virus may have on another.

Summary. 1. Yolk sac implants of a dba mouse mammary tumor protected the host embryos against vaccinia virus. A dosage of the virus which killed embryos of nontumor-bearing eggs in 3-4 days gave no evidence of toxicity in the embryos bearing the dba tumor 7 days after inoculation. Tests demonstrated that the virus was present in the fluids of these embryos. 2. Comparable tests with eggs bearing a C_3H mammary tumor indicated the embryos were only slightly protected against the virus, and yolk sac implants of a rat sarcoma gave negative results in this respect. 3. None of the 3 tumors affected the toxicity of a strain of influenza. 4. It is suggested that the dba tumor's protective effect on the embryos was due to the neutralization of the vaccinia virus by the tumor agent.

1. Turner, J. C., and Mulliken, B., *Cancer Research,* 1947, v7, 774.
2. Moore, A. E., *Cancer Research,* 1949, v9, 611.
3. ———, *Cancer,* 1951, v4, 375.
4. Klatt, O. A., and Taylor, A., *Cancer Research,* 1951, v11, 764.
5. Taylor, A., Carmichael, N., and Norris, T., *Cancer Research,* 1948, v8, 264.
6. Taylor, A., and Kynette, A., *A.A.A.S. Research Conference on Cancer,* 1945, 24.
7. ———, *Cancer Studies,* 1945, Univ. of Texas Pub. No. 4507, 33.
8. Taylor, A., and Carmichael, N., *Cancer Research,* 1947, v7, 78.
9. ———, *Cancer Research,* 1949, v9, 498.

Received June 10, 1953. P.S.E.B.M., 1953, v83.

Effect of Fatigue on Susceptibility of Mice to Poliomyelitis. (20456)

HERBERT E. ROSENBAUM* AND CARL G. HARFORD.

From the Division of Infectious Diseases, Department of Medicine, Oscar Johnson Institute for Medical Research, Washington University School of Medicine, St. Louis.

It is often observed that the paralysis of poliomyelitis is preceded by a period of exhausting physical exercise and the belief is prevalent that fatigue increases susceptibility to paralysis in persons already infected. Statistical studies(1-6) tend to confirm these clinical impressions but can be accepted only with reservations. For example, the use of non-paralytic cases for control(4) is subject to considerable error because of the difficulty in making an accurate clinical diagnosis in the absence of paralysis(7). Variables inherent in estimation of the degree of paralysis make judgment difficult on this basis alone(1-3). In one series, fatigue was associated with an increased incidence of paralysis in adults, but no significant difference was found in children(5). The association of increased paralysis with the distance of transportation of patients(6) may be due to other factors than fatigue.

To our knowledge, there has been only one previous report concerning the susceptibility of fatigued animals to experimental poliomyelitis(8). In these experiments, fatigue was induced in monkeys by forcing the animals to swim until exhausted. However, under these conditions it was difficult to distinguish between the effects of fatigue and chilling and use of monkeys limited the numbers of animals in each experiment. In our experiments, fatigue was induced by a technic that avoided immersion in water and the availability of mice permitted the use of relatively larger numbers of animals.

Materials and methods. In the beginning,

---

* Aided by a Fellowship from the National Foundation for Infantile Paralysis.

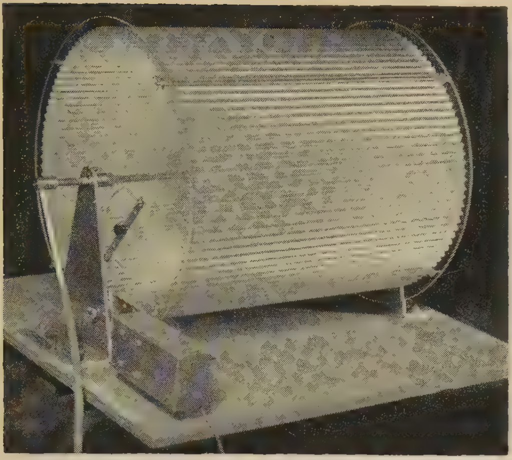


FIG. 1. Apparatus for inducing fatigue in mice. Dimensions of drum are 18 × 48 in. Corrugations are ⅜ in. wide. Ends of the drum are enclosed with plates of plexiglass ⅜ in. thick. Axle consists of a hollow tube of stainless steel with perforations to allow escape of air. Inlet for air is through a movable adaptor at one end. The drum is friction driven by contact between the edge of one plexiglass plate and a rubber stopper mounted on the drive shaft of a small motor. Rate of rotation is approximately 4 revolutions per min.

there was much difficulty devising an apparatus suitable for the purpose of inducing fatigue in mice. After a number of trials, the apparatus illustrated in Fig. 1 was developed. A drum of corrugated stainless steel was closed at both ends by discs of plexiglass, mounted on an axle, and rotated by a motor. Mice within the drum were forced to walk or run constantly since it was impossible for them to hold to the corrugated steel. For ventilation, a stream of compressed air was introduced into the drum through perforations in the hollow stainless steel axle. By this technic, mice could be exercised for many days at a constant rate and in such a way that there was no doubt of severe fatigue and yet the mice were not traumatized and did not die. After long periods of uninterrupted exercise, complete fatigue occurred and was recognized by the fact that the mice would roll in the drum instead of walking or running. In the experiments, the degree of fatigue was not allowed to reach this complete stage. The Lansing strain of poliomyelitis virus[†] was used in all the experiments. In preparation of each stock suspension of virus, the spinal cords and medullae of several mice showing paralysis were ground in a mortar with enough tryptose phosphate broth to make a 10% suspension. After centrifugation and culture for sterility, aliquots of the supernatant fluid were stored in a dry ice box and further dilution in broth was carried out on each aliquot just before use. Intracerebral injection of 10-fold dilutions into mice gave 50% infectivity titers of 10^{-4} to $10^{-4.5}$. The mice were of a single strain (CF1, Carworth Farms) and all mice of each experiment were young females of the same shipment.

In each experiment, the mice were divided into 2 groups. Mice of one group were exercised for 7 hours in the fatigue apparatus and immediately thereafter were injected intracerebrally under ether anesthesia with 0.03 ml of a dilution of viral suspension. Non-exercised mice in the other group serving as controls were injected at the same time with the same dilution of virus. Beginning the day after injection and continuing daily for a period of 14 days, the test groups of animals were exercised in the fatigue apparatus for a period of 8 hours. All animals were observed twice daily for paralysis over a period of 30 days. Animals in exercised groups showing paralysis were removed from the apparatus and not subjected to further exercise. Nevertheless, all such animals died. Mice found dead less than 48 hours after inoculation were thought to have died of trauma and were not included in the results. Mice found dead more than 48 hours after inoculation were considered to have died of viral infection even though paralysis had not been observed. Statistical analysis was carried out by the χ^2 method with a 4-fold table(9). In the experiments indicated, the Yates correction for small numbers was employed.[‡]

Results. Seven experiments were done in which a total of 223 mice were subjected to exercise and 328 mice were kept as controls. Viral dilutions were 1:5000 or 1:10,000. The incidence of paralysis and the fatalities in these experiments is shown in Table I and Fig. 2. It can be seen that larger numbers of

---

[†] Kindly furnished by Dr. Jonas Salk.

[‡] Advice concerning the statistical method was kindly given by Dr. Goldine Glaser, statistician of the Department of Neuropsychiatry.

TABLE I. Effect of Exercise on Incidence and Incubation Period of Experimental Poliomyelitis in Mice. 7 exp.

Dilution of virus	Exercised				Control				Chi square†	P
	In-jected	Para-lyzed	Died*	Mean time of sur-vival, days	In-jected	Para-lyzed	Died*	Mean time of sur-vival, days		
1: 5000	31	28	28	11.7	35	20	26	13.3	2.60‡	.1
1: ”	29	22	22	13.4	33	15	15	16.3	5.95	.02
1: ”	32	26	26	15.3	35	18	21	15.4	3.70	.05
1: ”	35	21	21	20.2	35	15	16	17.2	1.43	.2
1: ”	25	24	24	12.0	66	49	53	10.7	2.26‡	.1
1:10000	31	17	17	13.2	64	28	28	17.0	1.02	.3
1: ”	40	23	23	18.1	60	23	27	18.5	1.50	.2
	223	161	161		328	168	186		18.46	.02

* Includes all paralyzed mice and sometimes a few more that died without paralysis being observed.
† Based on number of deaths 2 to 30 days after inj.
‡ Yates correction for small numbers was used in these experiments.

exercised mice became paralyzed and died than did the control animals. The differences in each experiment were not great and, considered individually, the results of 5 of the 7 experiments were not within the range of statistical significance. However, the results were consistent and the accumulated values of χ^2 indicate that the observations as a whole are not likely to be due to chance.

The mean survival times of mice dying within 30 days are also shown in Table I. Since the differences in survival times between exercised and control animals were not considered significant, further details have not been recorded.

Discussion. It is important to note in these experiments that the differences between test and control groups were consistent. The difference, while small in the majority of the individual experiments, was statistically significant when the observations were considered as a whole. We believe, therefore, that the results are valid and that the small differences observed reflect the true influence of fatigue under these conditions.

The mechanism by which fatigue increases susceptibility to poliomyelitis is not known nor does it appear likely that evidence concerning the underlying processes can be obtained from statistical studies of human beings(10). However, the reproduction of this phenomenon in mice offers a model in which experiments concerning the nature of the increased susceptibility can be carried out. The possibility exists that exercise affects the adrenal cortex causing increase or decrease of hormones which in turn influence susceptibility to this viral infection. Supporting this concept are reports that administration of adrenal or pituitary hormones to experimental animals may increase their susceptibility to experimental infection with poliomyelitis virus (11-15).[§]

Summary. In order to determine the effect of fatigue on susceptibility to experimental poliomyelitis, mice were forced to run in a revolving drum during the day preceding and for 8 hours each day subsequent to the intra-

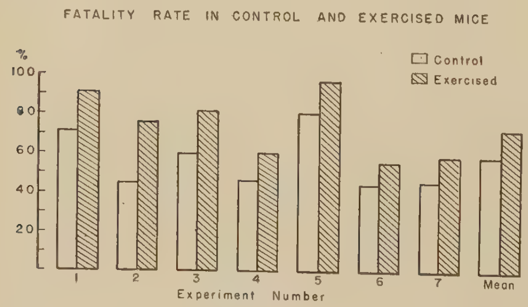


FIG. 2.

[§] Because of reports indicating that fatigue of nerve cells is associated with depletion of Nissl substance(16-19), an attempt was made to correlate this phenomenon with increased susceptibility to infection with poliomyelitis virus. Paraffin sections were prepared of spinal cords of normal and fatigued mice and stains for Nissl substance were done carefully by several methods. However, the amounts of Nissl substance in the nerve cells of a single section were found to vary so greatly that no conclusions could be drawn as to its depletion in fatigued mice.

cerebral injection of the Lansing strain of virus. Control animals inoculated at the same time with the same amount of virus were allowed to rest in their cages. In all 7 experiments, the incidence of the disease as measured both by paralysis and by death was greater in the exercised animals than in the resting controls.

Valuable technical assistance in this work has been rendered by Esther Parker and Alice Hamlin.

1. Russell, W. R., *Brit. M. J.*, 1947, v2, 1023.
2. Hargreaves, E. R., *Brit. M. J.*, 1948, v2, 1021.
3. Russell, W. R., *Brit. M. J.*, 1949, v1, 465.
4. Horstmann, D. M., *J. Am. Med. Assn.*, 1950, v142, 236.
5. Albrecht, R. M., and Locke, F. B., *J. Am. Med. Assn.*, 1951, v146, 769.
6. Brahdy, M. B., and Katz, S. H., *J. Am. Med. Assn.*, 1951, v146, 772.
7. Kilham, L., Levens, J., and Enders, J. F., *J. Am. Med. Assn.*, 1949, v140, 934.
8. Levinson, S. O., Milzer, A., and Lewin, P., *Am. J. Hyg.*, 1945, v42, 204.
9. Hill, A. B., *Principles of Medical Statistics*, London, The Lancet Limited, 3rd edition, 1945, 92.
10. Perla, D., and Marmorston, J., *Natural Resistance and Clinical Medicine*, Boston, Little, Brown and Co., 1941, 1137.
11. Shwartzman, G., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 835.
12. Ainslie, J. D., Francis, T., Jr., and Brown, G. C., *J. Lab. and Clin. Med.*, 1951, v38, 344.
13. Foster, C., Sigel, M. M., Henle, W., and Stokes, J., Jr., *J. Lab. and Clin. Med.*, 1951, v38, 359.
14. Shwartzman, G., and Fisher, A., *J. Exp. Med.*, 1952, v95, 347.
15. Findlay, G. M., and Howard, E. M., *J. Pharm. and Pharmacol.*, 1952, v4, 37.
16. Mann, G., *J. Anat. and Physiol.*, 1894, v29, 100.
17. Faure, C., and Soula, C., *Compt. rend. Soc. Biol.*, 1913, v74, 350.
18. Hydén, H., *Acta Physiol. Scand.*, 1943, v6, Supp. 17.
19. Barr, M. L., and Bertram, E. G., *J. Anat.*, 1951, v85, 171.

Received June 11, 1953. P.S.E.B.M., 1953, v83.

Oral Administration of Co^{60} Vit. B_{12} in Tropical Sprue and Hepatic Cirrhosis and Diarrhea.* (20457)

LEO M. MEYER, RAMON M. SUAREZ, JR., ROBERTO BUSO, JUAN SABATER, AND RAMON M. SUAREZ.

From the Laboratories of the Fundacion de Investigaciones Clinicas and the Hospital Mimiya, Santurce, P. R.

Smith reported that in the stools of rats fed Co^{60} vit. B_{12} the radioactivity is present in a conjugated or degraded form of the vitamin but not as ionized cobalt(1). Rosenblum and his coworkers regarded this as unlikely and concluded that vit. B_{12} is destroyed in the feces, probably by bacterial action(2). Barbee and Johnson, by means of radioautographs of paper strip chromatograms, showed that the feces of rats contained considerable ionic Co^{60} after oral administration of the labelled vitamin, and that all the radioactivity in the feces after injection is in this form(3).

The present study was undertaken to determine fecal excretion of radioactive cobalt following oral administration of Co^{60} vit. B_{12}

TABLE I. Radioactivity of Stools Following Oral Administration of 0.5 μg of Co^{60} Vit. B_{12} to 4 Normal Subjects.

Patient	Age	Total fecal excretion of Co^{60} (%)
D.	26	78
O.	30	31
R.	30	39
S.	33	50

---

* Radioactive vit. B_{12} was supplied by Merck and Co., Rahway, N. J. Expenses for these studies were partially defrayed by grants from the Squibb Institute for Medical Research, New Brunswick, N. J. and the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

TABLE II. Radioactivity of Stools Following Oral Administration of 0.5 μg of Co^{60} Vit. B_{12} and Folic Acid to 6 Patients with Tropical Sprue in Relapse and 1 Person with Cirrhosis of the Liver and Diarrhea.

Patient	Age	Free HCl	.5 μg Co^{60} B_{12} (%)	.5 μg Co^{60} B_{12} + 1 mg folic acid (%)	.5 μg Co^{60} B_{12} + 5 mg folic acid (%)	.5 μg Co^{60} B_{12} + 10 mg folic acid daily for 7 days (%)	.5 μg Co^{60} B_{12} + 10 mg folic acid daily for 3 mo (%)
M.R.	25	+	46				
A.C.	60	+	32				
R.H.	62	+	34				
P.V.	25	+	19			57	
V.L.	65	—	59 66*	83	18	88	
J.S.	15	+	91 67†	56	24	3	1
C.L.	30	+	90	100	91	33	

* Repeated after patient received .5 μg Co^{60} B_{12} and 1 mg folic acid.
† ” ” ” ” ” ” ” 5 mg ” .

to 6 patients with tropical sprue in relapse and one subject with hepatic cirrhosis and diarrhea. Free HCl was present in the gastric juice in all instances except one (V.L.). All cases showed a flat glucose tolerance curve following ingestion of dextrose. The labelled vitamin had a specific activity of 0.245 μc/μg. In each experiment the patient received 0.5 μg in 250 ml of water in the morning. Breakfast was withheld until noon. During the period of observation the patients received an inadequate diet, the socalled "preliminary sprue diet" which is low in proteins and vitamins, and consists mainly of rice, beans, root and starchy vegetables, and small amounts of milk and cod-fish. Stool collections and measurements were made in the manner previously described(4). When fecal radioactivity returned to background 3 of the patients received one and 5 mg of folic acid orally with the radioactive vit. B_{12}. Four of the subjects were given 10 mg of folic acid, orally, daily, for one week and then treated with one dose of Co^{60} vit. B_{12}. One patient received 10 mg of folic acid orally, daily, for 3 months and then a single dose of the labelled vitamin. Four normal young adults on regular hospital diets served as controls. No gastric extractions were performed on them.

Results. Summaries of the data are presented in Tables I and II. Patients M.R., A.C., and R.H. refused to cooperate after the preliminary survey and were lost for future study. Under the conditions of the experiment the normal subjects excreted 31 to 78% activity of the orally administered dose. Patients with tropical sprue in relapse excreted 19 to 91%. Following the simultaneous ingestion of 1 mg of folic acid and 0.5 μg of labelled vit. B_{12} there was no change in fecal radioactivity in 2 cases and reduction of about 30% in a third (J.S.). This latter patient showed a suboptimal reticulocytosis and hematologic improvement following the single combined dose. Oral administration of a second dose of Co^{60} vit. B_{12} to this patient was followed by an excretion of 67% of ingested activity. The ingestion of 5 mg of folic acid and 0.5 μg of radioactive vit. B_{12} to 3 subjects resulted in high fecal activity in one (C.L.) and striking reductions in 2 (V.L. and J.S.). The oral administration of 10 mg of folic acid, orally, daily, for one week, followed by a single dose of 0.5 μg of labelled vit. B_{12} resulted in elevated fecal activity in 2 patients (P.V. and V.L.) and low activity in the others (J.S. and C.L.). There was an almost complete absence of stool radioactivity in the patient who was treated with 10 mg of folic acid, orally, daily, for 3 months and received 0.5 μg of Co^{60} vit. B_{12} at the end of that period.

Summary. From the data it is apparent that no conclusion can be reached regarding interpretation of Co^{60} activity in the stool following oral administration of 0.5 μg of radioactive vit. B_{12} because of the wide normal range which overlaps with that of patients with tropical sprue in relapse and one subject with hepatic cirrhosis and diarrhea. No consistent pattern of change was observed when folic acid was added. The variable results do not bear any relationship to the presence or absence of free HCl in gastric juice.

1. Smith, E. L., *Brit. Med. Bull.*, 1952, v8, 203.

2. Rosenblum, C., Chow, B. F., Condon, G. P., and Yamamoto, R. S., *J. Biol. Chem.*, 1952, v198, 915.

3. Barbee, K. W., and Johnson, B. C., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 720.

4. Meyer, L. M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 490.

Received June 15, 1953. P.S.E.B.M., 1953, v83.

Accentuation of Human Diabetes by "Pituitary Growth Hormone".* (20458)

LAURANCE W. KINSELL, HARRY E. BALCH, AND GEORGE D. MICHAELS.
(With the technical assistance of June Bilisoly, George Fukayama, Eleanor Kipp,
Florence Olson, and Sadie Smyrl)

*From the Institute for Metabolic Research of the Highland Alameda County Hospital,
Oakland, Calif.*

Young demonstrated that extracts of the anterior pituitary, which possessed growth-promoting effects, were also diabetogenic(1). Subsequently it has been shown that more highly purified growth hormone preparations retain the property of diabetogenicity in experimental animals(2). It appears to be well established that these diabetogenic effects are not related to ACTH contamination. In studies with "pituitary growth hormone preparations" in human subjects, under chemically controlled metabolic ward conditions extending over the period 1948 to 1953, we have failed to note any diabetogenicity of the preparations used. However, except in one subject, we have also failed to obtain growth-promoting effects(3).

The present study was carried out in a 60-year-old male patient with diabetes mellitus of at least 5 years' duration, and of some severity. It was designed to determine whether "highly purified pituitary growth hormone" would have either growth-promoting or diabetogenic effects under the experimental conditions used.

Experimental. The patient was well equilibrated on a formula diet containing 119 g of protein, 130 g of fat, and 184 g of carbohydrate. The formula was ingested at hourly intervals during the period 6:00 a.m. to 10:00 p.m. Urine and stool collections and chemical and other determinations were carried out as described elsewhere(4). The growth hormone was administered by slow intravenous drip in a solution containing 2 g of potassium chloride and 2 g of sodium chloride per liter. In order to make for precise constancy of conditions throughout the study, the intravenous sodium-potassium chloride solution was administered during the control as well as the treatment periods. Growth hormone (Armour, Lot No. 491132) was administered during the treatment period in a dose of 100 mg equivalents per day, in one liter of solution at a rate of 180 cc per hour. Forty units of N.P.H. insulin daily were given throughout. The assay of the growth hormone provided by Drs. Hays and Steelman and their associates of the Armour Laboratories was as follows: potency—49.5 ± 10 mg stand./vial; T.S.H. —.04 ± .02 USP u/mg; oxytocin — <.8 u/vials; fill—50 mg; ACTH — <.01 u/mg; pH—9.4.

Experimental findings. As shown in Fig. 1 and 2, the effects of "pituitary growth hormone" in this patient were as follows: 1. Blood and urine sugars were elevated during and following the administration of the hormone. The daily urinary sugar excretion

---

* This work was supported by grants from the Armour Laboratories and the National Institutes of Health.

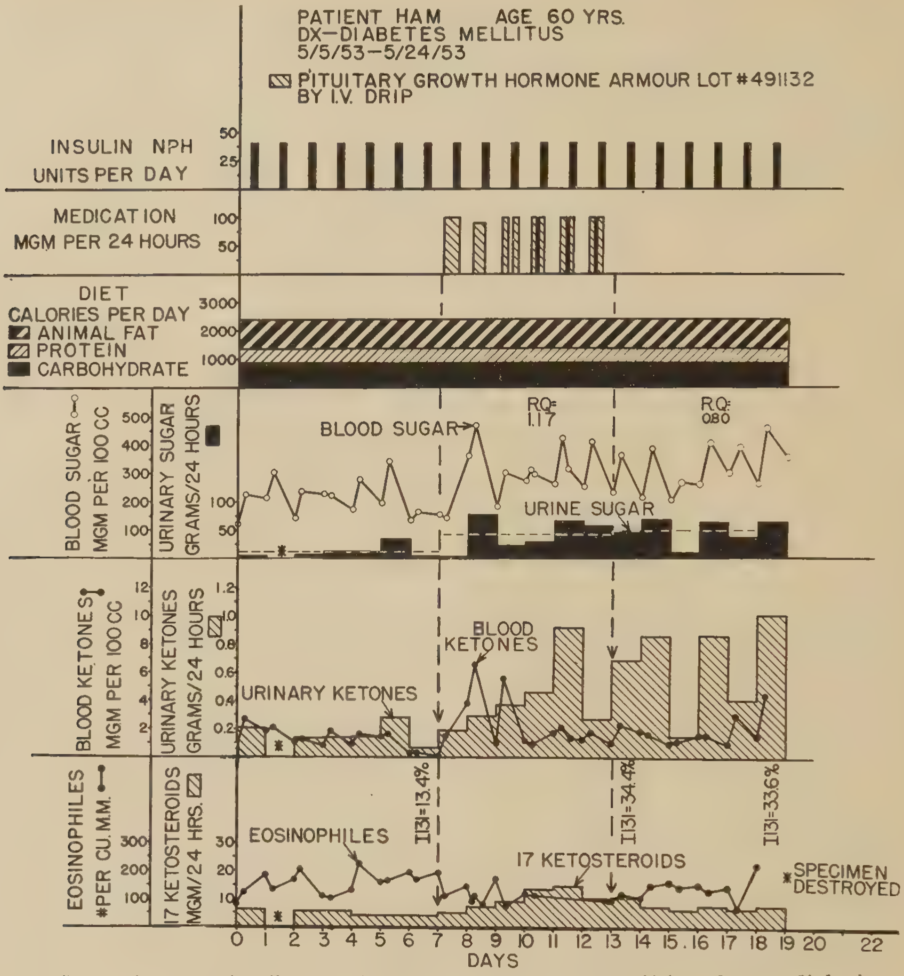


FIG. 1. Diabetogenic effects of "pituitary growth hormone" in a human diabetic.

during pre-treatment averaged 11.8 g per day; 43.0 g per day during hormone administration; and 49.0 g per day following hormone administration.

2. A significant but unimpressive increase in blood and urinary ketones occurred during and following the administration of pituitary growth hormone. The increase is much less than has been noted in similar diabetic patients to whom ACTH has been administered under essentially identical experimental conditions(5).

3. There was a significant, but not marked, elevation of 17-ketosteroids during pituitary growth hormone administration, with a less than complete return to base line levels when the hormone was stopped.

4. A probably significant, but not impressive, decrease in circulating eosinophils occurred during the administration of pituitary growth hormone. (In one other study in a non-diabetic, the administration of one unit of ACTH by slow intravenous drip caused a more marked fall in eosinophils than that noted in this study.)

5. An increase in iodine-131 uptake from 13.4% to 34% was noted during and following the administration of the hormone. (The patient showed some clinical evidence of hypothyroidism prior to treatment. This was part of the basis for his selection as a candidate for the study.)

6. A respiratory quotient of 1.17 during the hormone administration. (In diabetic pa-

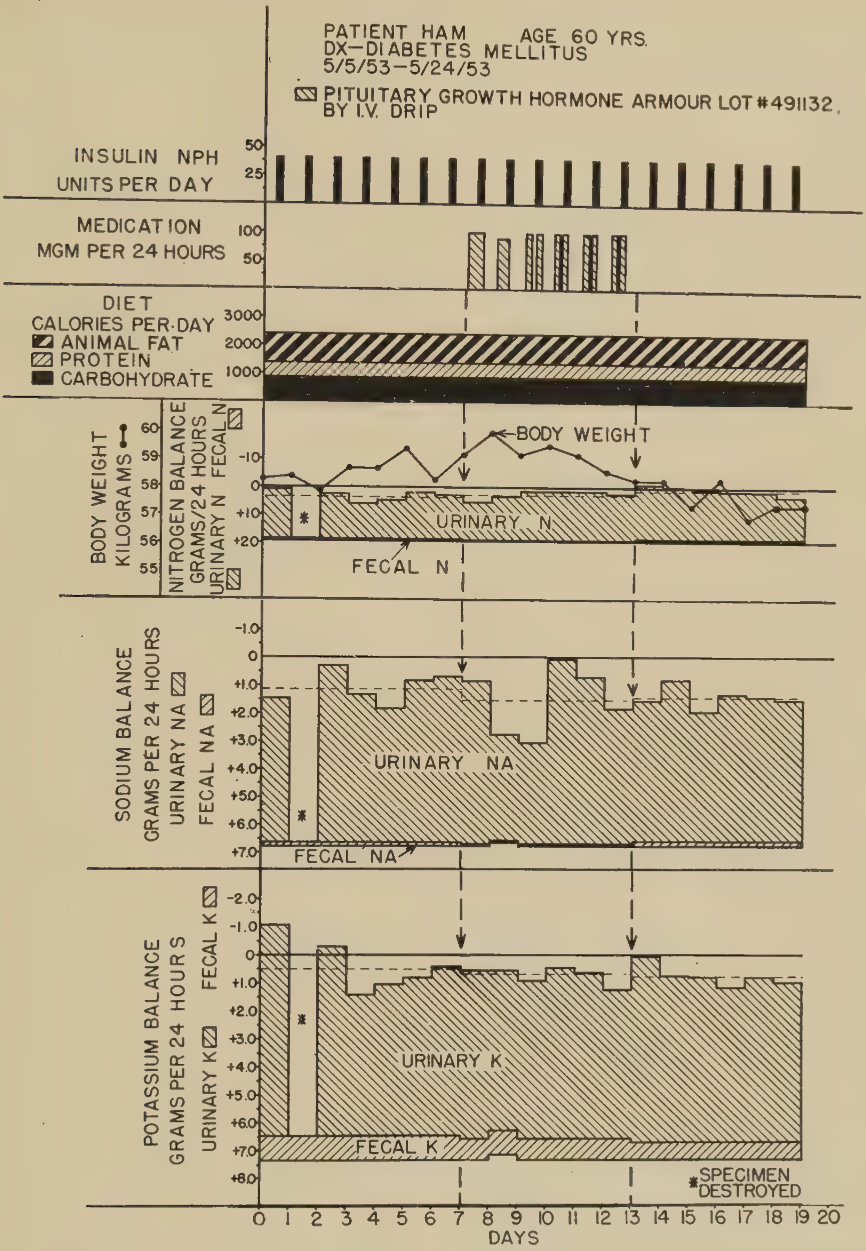


FIG. 2. Effects of "pituitary growth hormone" upon nitrogen, sodium and potassium balances in a human diabetic.

tients in whom the severity of the diabetes has been accentuated with ACTH or cortisone, without exception, the respiratory quotient has been depressed below 0.70.)

7. The average daily nitrogen excretion prior to growth hormone was 15.97 g, during —16.90 g, and following—18.15 g.

8. There was a net retention of 0.639 g of sodium per day during, and 0.385 g following growth hormone, as compared to the pretreatment control period.

9. There was a slight net retention of potassium.

The changes in body weight were probably referable to the combined effects of changes in fluid balance, changes in nitrogen balance, and secondarily to the loss of the sugar in the urine.

Discussion. From the foregoing, it appears that the administration of "pituitary growth hormone" to a diabetic patient results in marked accentuation of the diabetic state. It is possible that the thyrotropin and/or adrenocorticotropin present in the preparation may have played some role in the hyperglycemia and in the increased glycosuria. It seems probable, however, that the role played by these 2 factors was small for the following reasons: 1. The relatively slight change in circulating eosinophils and urinary 17-ketosteroids; 2. Slight increase in nitrogen excretion; 3. Elevation of the respiratory quotient above one, rather than below 0.7. (The latter occurs consistently in diabetics receiving ACTH)(5); 4. Decreased rather than increased potassium excretion; 5. Decreased oxygen utilization.

Summary. Administration of "highly purified pituitary growth hormone" to a diabetic patient, under chemically controlled conditions, resulted in increased hyperglycemia and glycosuria, in minimal increase in blood and urinary ketones, and in minimal change in the nitrogen balance. The data support the concept that the increased hyperglycemia and glycosuria are probably attributable to diminished carbohydrate utilization rather than increased neoglucogenesis. The diminished utilization in turn may be referable to diminished endogenous insulin production as the result of the exhaustion of the beta cells of the islets.

Grateful acknowledgement is made to Dr. Hays and Dr. Steelman of the Armour Laboratories for supplies of pituitary growth hormone, and to the Borden Co. for supplies of powdered whole milk and instant coffee.

---

1. Young, F. G., *Biochem. J.*, 1945, v39, 515.

2. a. ———, *J. Clin. Endocrin.*, 1951, v11, 531. b. Milman, A. E., DeMoor, P., and Lukens, F. D. W., *Am. J. Physiol.*, 1951, v166, 354.

3. Kinsell, L. W., Margen, S., Partridge, J. W., Michaels, G. D., Balch, H. E., and Jahn, J. P., submitted for publication to *J. Clin. Endocrin. and Metab.*

4. a. Michaels, G. D., Margen, S., Liebert, G., and Kinsell, L. W., *J. Clin. Invest.*, 1951, v30, 1483. b. Michaels, G. D., Anderson, C. T., Margen, S., and Kinsell, L. W., *J. Biol. Chem.*, 1949, v180, 175. c. Giragossintz, G. D., Davidson, C., and Kirk, P. L., *Mikrochemie*, 1936, v21, 21. d. Kirk, P. L., *Indust. and Engin. Chem.*, Analyt. Ed., 1936, v8, 223.

5. a. Kinsell, L. W., Margen, S., Michaels, G. D., Reiss, R., Frantz, R., and Carbone, J., *J. Clin. Endocrinol.*, 1950, v10, 815. b. Margen, S., Michaels, G., Mudrich, C., Barton, H., Reiss, R., and Kinsell, L., *J. Clin. Endocrin.*, 1950, v10, 834. c. Kinsell, L. W., Margen, S., Boling, L., Michaels, G. D., and Partridge, J. W., *J. Clin. Endocrin.*, 1951, v11, 773. d. Kinsell, L. W., Michaels, G. D., Margen, S., Partridge, J. W., Boling, L., and Balch, H. E., (submitted for publication to *J. Clin. Endocrin. and Metab.*).

Received June 22, 1953. P.S.E.B.M., 1953, v83.

Identification of Trypsinogen in the Lipotropic Fraction.* (20459)

R. J. PEANASKY† AND M. LASKOWSKI.

From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wisc.

Haanes and György(1) analyzed the lipotropic fraction prepared by Bosshardt *et al.*(2) from the residue remaining after the extraction of pancreas for insulin, and identified trypsin and trypsin inhibitor as components of this fraction. Furthermore, they postulated that enterokinase is capable of overcoming the action of trypsin inhibitor on trypsin. Mars, Peanasky, and Laskowski(3) studied a system composed of crystalline trypsin, crystalline pancreatic trypsin inhibitor and highly purified enterokinase, but were unable to confirm the hypothesis of a new activity of enterokinase. In order to explain the discrepancy between their results and those of Haanes and György(1), Mars *et al.*(3) tentatively postulated that, in addition to the components identified by Haanes and György, the lipotropic fraction contained trypsinogen. Through the courtesy of Dr. L. A. Kazal of Sharp and Dohme, Inc., we recently received a small amount of lipotropic fraction and analyzed it for trypsinogen.

---

* Aided by a research grant from the National Institute of Health, U. S. Public Health Service.

† Public Health Service Research Fellow of National Heart Institute. Present address: Institute for Enzyme Research, University of Wisconsin, Madison, Wisc.

Experimental. The lipotropic fraction was treated according to the procedure of Kunitz and Northrop(4) devised for the separation of trypsin and/or trypsinogen from the trypsin inhibitor. 1.250 g of the lipotropic fraction was suspended in 0.0025 N HCl and 0.1 N HCl was added dropwise until the pH of the mixture was 3.0. The insoluble material was centrifuged off and discarded. The yellow liquid, 8 ml, was mixed with 8 ml of 5% trichloroacetic acid (TCA), left for one hour at room temperature, and centrifuged. The supernatant was discarded. The precipitate was washed with 2 ml of 2.5% TCA, was then dissolved in 40 ml of 0.0025 N HCl and left at room temperature for 30 minutes. Solid ammonium sulfate was added (24 g/100 ml) to attain 0.4 saturation. The precipitate which formed was centrifuged off and discarded. To the supernatant, ammonium sulfate (36.1 g/100 ml) was added to attain 0.8 saturation, the resulting suspension was centrifuged and the supernatant was discarded. The precipitate, referred to as 0.8 S.A.S. (saturation with ammonium sulfate) precipitate, was dissolved in 0.0025 N HCl and was assayed, according to the method of Kunitz (5). To some tubes $CaCl_2$ was added, since Gorini(6) and Nord and coworkers(7,8) had shown that calcium exerted a stabilizing effect on trypsin.

A rapid activation of trypsinogen was performed at 37°C, at pH 7.6 (Table I, Exp. 1). All tubes contained 0.8 S.A.S. fraction in a final concentration equivalent to an optical density of 3.24 at 280 μ ($E^{1\,cm}_{280} = 3.24$) and 0.1 M borate buffer, pH 7.6. Tubes 1 and 2 contained enterokinase, final concentration 10 E.K.U. (enterokinase units)(9) per ml. Tubes 1 and 3 contained $CaCl_2$, final concentration 0.01 M. At the indicated times 0.1 ml aliquots were withdrawn from each tube, added to a mixture of 0.9 ml of water and one ml of 1% casein solution, and incubated for 20 minutes. Proteolytic activity was determined by spectrophotometric assay of the trichloroacetic-acid-soluble digestion products (5). Control tubes containing an equivalent amount of enterokinase and control tubes containing casein only were incubated simultaneously. The amounts of enterokinase used were too low to exhibit detectable proteolytic activity. The 2 series of controls gave identical results. All figures shown in Table I were corrected for the values of the corresponding controls.

TABLE I. Effect of Enterokinase on Proteolytic Activity of 0.8 S.A.S. Fraction.

	$E^{1\,cm}_{280}$		
	0	½ hr	3 hr
Exp. 1, pH 7.6, 37°			
Tube 1, enterokinase, Ca	.520	.610	.505
2, ” , no Ca	.370	.405	.185
3, no enterokinase, Ca	.031	.030	.027
4, ” ” , no Ca	.034	.043	.020
Exp. 2, pH 5.6, 5°	0	5 hr	24 hr
Tube 1, enterokinase, Ca	.118	.170	.280
2, ” , no Ca	.044	.146	.298
3, no enterokinase, Ca	.014	.015	.012
4, ” ” , no Ca	.010	.013	.006

Under the conditions described, the activation occurred very rapidly; the highest peak of proteolytic activity being reached in Tubes 1 and 2 after 30 minutes of incubation. In Tube 1, containing calcium, proteolytic activity decreased slowly during the period of 5 hours. In Tube 2, without calcium, the proteolytic activity decreased sharply, indicating that under these conditions denaturation and self-digestion of trypsin occurred very rapidly(10). Contrary to expectations, only a small part of the potential proteolytic activity was present in the tubes which did not contain enterokinase, indicating that the 0.8 S.A.S. fraction contained predominantly trypsinogen with relatively little trypsin.

An experiment was then performed in which the 0.8 S.A.S. fraction was subjected to a slow activation at 5°, at pH 5.6 (Table I, Exp. 2). All tubes contained 0.8 S.A.S. fraction, final concentration $E^{1\,cm}_{280} = 0.315$, and 0.1 M acetate buffer, pH 5.6. Tubes 1 and 2 contained enterokinase, final concentration 1 E.K.U. per ml. Tubes 1 and 3 contained $CaCl_2$, final concentration 0.01 M at the indicated time 0.5 ml aliquots were withdrawn and assayed for tryptic activity as before. Control tubes, analogous to those of Exp. 1 were incubated simultaneously. All figures were corrected for the values of the corresponding controls.

Under the conditions described, a steady increase in proteolytic activity during the period of 24 hours was observed in Tubes 1 and 2, containing enterokinase. At 5° and pH 5.6, the rate of denaturation and self-digestion of trypsin was very low(10), and therefore, the protective effect of calcium was not evidenced.

In order to determine whether or not the zymogen was really trypsinogen, it was decided to investigate the action of soy bean trypsin inhibitor on the activated enzyme. Kunitz(5) showed that soy bean trypsin inhibitor reacts stoichiometrically with trypsin, but forms a dissociable complex with chymotrypsin. To 4 ml of solution of 0.8 S.A.S. fraction ($E^{1\,cm}_{280} = 1.2$) were added 4 ml of 0.2 M borate buffer, pH 7.6 and 2 ml of enterokinase solution containing 10 E.K.U. per ml. The mixture was incubated for one hour at 37°C, and five 0.5 ml aliquots were then withdrawn and were mixed with solutions containing 0, 13, 65, 130, and 650 γ of soy bean inhibitor. After 5 minutes casein was added, and the tubes were incubated for 20 minutes. A series of control tubes containing corresponding amounts of inhibitor was incubated simultaneously. For the tube containing no inhibitor, the corrected reading was 0.285. For the tubes containing inhibitor corrected readings varied from 0.050 to 0.040, indicating that about 86% of the proteolytic activity was inhibited, regardless of the amount of inhibitor used. No attempts were made to identify the residual proteolytic activity. Considering the origin and the treatment to which it was subjected, it would appear most likely that chymotrypsin was responsible for the residual proteolytic activity. The results of this experiment agree with the conclusion of Haanes and György(1) that trypsin represented the major proteolytic component of the activated lipotropic fraction.

Summary. The postulation that the lipotropic fraction prepared by Bosshardt *et al.* (2) contained trypsinogen has been confirmed by a direct analysis. In view of this finding, it is no longer necessary to ascribe an additional new activity to enterokinase.

---

1. Haanes, M. L., and Gyorgy, P., *Am. J. Physiol.,* 1951, v166, 441.

2. Bosshardt, D. K., Ciereskо, L. S., and Barnes, R. H., *Am. J. Physiol.,* 1951, v166, 433.

3. Mars, P. H., Peanasky, R. J., and Laskowski, M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 384.

4. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.,* 1936, v19, 991.

5. Kunitz, M., *J. Gen. Physiol.,* 1947, v30, 291.

6. Gorini, L., *Biochim. Biophys. Acta,* 1951, v7, 318.

7. Bier, M., and Nord, F. F., *Arch. Biochem. Biophys.,* 1951, v33, 320.

8. Duke, J. A., Bier, M., and Nord, F. F., *Arch. Biochem. Biophys.,* 1952, v40, 424.

9. Kunitz, M., *J. Gen. Physiol.,* 1939, v22, 447.

10. Northrop, J. H., Kunitz, M., and Herriot, R. H., *Crystalline Enzymes,* 2nd edition, Columbia University Press, 1948.

Received June 22, 1953. P.S.E.B.M., 1953, v83.

Antigenic Dissimilarity of Streptokinases. (20460)

LOUIS WEINSTEIN.

From the Haynes Memorial and the Evans Memorial, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine, Boston, Mass.

The demonstration of an increase in the capacity of serum to inhibit streptokinase has been assumed to indicate recent experience with the beta hemolytic *Streptococcus*(1-4). Anti-fibrinolytic activity, however, may not be due entirely to the presence of specific antibody. Since the dissolution of clotted plasma is actually carried out by proteolytic enzymes in the plasma, an increase in the quantity of non-specific antiprotease may be responsible, in part, for an antifibrinolytic effect(5,6). A deficiency of the lytic factor in serum may produce the same effect(7,8). Most of the studies of antifibrinolysis have until recently been complicated, therefore, by these non-specific factors. Kaplan(9), however, has devised a quantitative serological method which minimizes the effects of antiprotease and deficiency of lytic factor.

In carrying out determinations of antifibrinolytic activity of serum, it has been customary to use the streptokinase produced by one strain of beta hemolytic *Streptococcus*. This has been based on the assumption that all streptokinases of Group A *Streptococcus* are antigenically similar. Van Deventer(10), on the basis of an investigation of 40 strains of streptococci, concluded that the fibrinolysins elaborated by all of them were of one immunological type. These observations were confirmed recently by Kaplan(11) who demonstrated the similarity of streptokinases of Group A, C, and G streptococci. In contrast, Mote, Massell, and Jones(12) found that the resistance of plasma clots varied with the fibrinolysins derived from different strains. They attributed these results to immunological differences in antifibrinolysin.

The purpose of the present study was to reinvestigate the problem of the antigenic similarity of streptokinase, using the quantitative technic of Kaplan(9). It was found that the streptokinases produced by different strains of the beta hemolytic *Streptococcus* were antigenically dissimilar.

Methods. The 10 strains of beta hemolytic streptococci used for the preparation of streptokinase were isolated from patients with scarlet fever, with the exception of strain CO which was obtained from Dr. Tillett; all belonged to group A. The organisms were grown in heart infusion-yeast-blood broth for 24 hours and the fibrinolysin prepared by alcoholic precipitation, according to the method of Garner and Tillett(13). Titrations of fibrinolytic activity of each strain were repeated before each group of sera was examined for antistreptokinase activity. The procedure of Kaplan(9) was employed for estimation of antifibrinolytic activity and all determinations were carried out at least twice.

The serums used in this study were obtained from patients with untreated scarlet fever. Group A *Streptococcus pyogenes* was isolated from the pharynges of all these individuals and all developed high antistreptolysin titers. The sera were first assayed for antifibrinolysin using streptokinase prepared from strain CO, the organism with which all antistreptokinase titrations had been carried out in this laboratory for several years. On the basis of these results, the cases were divided into 2 groups; those showing a "normal" titer (14 strains) and those with an "elevated" titer of antistreptokinase (11 strains). Although it is difficult to establish the exact point beyond which the antistreptokinase activity may be considered to be increased, levels over 125 (that is, 179 units or greater), were considered by the Commission on Acute Respiratory Diseases to be elevated (14).

Results. In Table I are presented the results of the titrations of sera which appeared to contain a "normal" antistreptokinase content when examined with the fibrinolysin of Strain CO. As can be seen from the data, only 2 (No. 5 and 8) reacted with the 10 streptokinases so that all the determinations were less than 2 tubes apart. The remaining

TABLE I. Antistreptokinase Titers of Serums Using Different Streptokinases. Low-titer serums.

Serum No.	Antistreptokinase titer — Strain of strep. from which streptokinase was prepared: CO	12	23	18	5	15	17	8	6	9	No. of tubes difference
1	83	<25	<25	83	25	36	<25	<25	36	<25	4
2	56	<25	<25	56	179	<25	36	56	56	<25	6
3	83	<25	<25	25	<25	36	56	<25	36	<25	3
4	56	83	417	83	<25	278	36	36	179	<25	8
5	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	0
6	25	36	<25	278	36	<25	278	36	<25	<25	7
7	36	25	<25	36	<25	36	25	83	<25	36	3
8	<25	<25	<25	25	<25	<25	36	<25	36	<25	1
9	<25	25	<25	<25	56	<25	56	<25	83	<25	3
10	56	278	179	83	<25	278	83	56	179	278	7
11	36	125	83	83	36	36	36	179	179	56	4
12	25	36	125	125	36	179	36	56	56	56	5
13	125	<25	<25	179	83	<25	56	<25	36	36	6
14	125	125	125	125	125	125	83	125	125	25	4

TABLE II. Antistreptokinase Titers of Serums Using Different Streptokinases. High-titer serums.

Serum No.	Antistreptokinase titer — Strain of strep. from which streptokinase was prepared: CO	12	23	18	5	15	17	8	6	9	No. of tubes difference
15	278	278	179	278	625	278	278	83	179	56	6
16	179	56	125	179	179	83	125	56	56	25	5
17	179	<25	<25	83	36	<25	25	<25	36	<25	6
18	417	278	417	36	<25	417	83	179	83	56	8
19	179	417	417	179	83	278	83	179	125	83	4
20	179	125	278	278	278	179	278	83	125	83	3
21	179	125	83	417	125	56	179	179	83	179	5
22	278	56	<25	56	25	56	25	125	56	25	7
23	278	278	83	625	278	83	179	179	83	<25	9
24	417	278	278	179	83	278	125	278	179	83	4
25	417	83	56	56	125	83	83	36	83	36	6

12 gave variable results with the different kinases, the titrations ranging from 3 to 8 tubes apart. Although all of the serums had been classified as showing no elevation of antistreptokinse with CO, several of them (No. 2, 4, 6, 10, 11, 12, and 13) appeared to contain an increased quantity of antibody when tested with other fibrinolysins. While the remaining 5 sera showed variations in titer, all of them were within the range of what is considered to be normal.

The discrepancies between antistreptokinase titers determined by using different streptococcal fibrinolysins were even more pronounced when sera originally considered to contain an increased quantity of antibody against the CO kinase were examined for their ability to inactivate lysins prepared from other strains. The smallest difference in titers of any single serum was 3 and the largest 9 tubes.

Although all of the sera had been considered to have an elevated quantity of antibody when first examined, using the CO streptokinase, all also revealed only a "normal" content of anti-fibrinolysin when tested with the enzyme elaborated by other strains of streptococci. In most instances the differences in titer for each serum fell well outside the limit of experimental error of 2 tubes. It is striking that with no other lysin than that derived from strain CO did the sera give consistently elevated anti-streptokinase levels (Table II).

Discussion and summary. The results obtained in this study indicate that streptokinases derived from different strains of Group A *Streptococcus pyogenes* are antigenically dissimilar. Since Kaplan's method of determining antifibrinolysin(9) has been claimed to minimize the non-specific effects of antiprotease and inadequacy of lytic substance in the blood, the discrepancies in quantity of antifibrinolysin observed in the present investigation probably cannot be attributed to these factors. It is a well known fact that an increase in antistreptokinase occurs much less frequently than a rise in antistreptolysin in streptococcal infections. Rantz, Boisvert, and Spink(15) and the Commission on Acute Respiratory Diseases and Kaplan(16) have attributed this to the marked variations in the ability of different strains of streptococci to produce fibrinolysin. On the basis of the observations made in the present study it would appear likely also that the relative infrequency of increase in antistreptokinase after streptococcal infection may be related to the use of a fibrinolysin antigenically different from that elaborated by the strain of organism responsible for the disease.

The reason for the difference in the results obtained by Kaplan(11) and those presented in this paper are not readily apparent. They may be related, however, to the fact that, by chance, the 3 lysins which Kaplan used happened to be antigenically the same. With an increase in the number of kinases examined, the possibility of detecting immunologic differences probably was increased.

If, as the data presented in this paper indicate, streptococcal fibrinolysins are antigenically dissimilar, the determination of antistreptokinase titers probably is of little value in the diagnosis of streptococcal infection. A low antibody content may be related not to lack of immune response but to the fact that the fibrinolysin used in carrying out the test was immunologically different from that produced by the organism which infected the patient. It is evident that elevation of antistreptolysin is of much greater significance in the recognition of recent invasion by the beta-hemolytic *Streptococcus*.

Conclusions. 1. Streptokinases produced by different strains of Group A beta-hemolytic *Streptococcus* are antigenically different. 2. Determination of antistreptokinase titers is probably of little value in the diagnosis of streptococcal infection.

---

1. Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, v58, 485.
2. Tillett, W. S., Edwards, L. B., and Garner, R. L., *J. Clin. Invest.*, 1934, v13, 47.
3. Tillett, W. S., *Bact. Rev.*, 1938, v2, 161.
4. Mote, J. R., and Jones, T. D., *J. Immunol.*, 1941, v41, 61.
5. Mirsky, I. A., *Science*, 1944, v100, 198.
6. Kaplan, M. H., *J. Clin. Invest.*, 1946, v25, 337.
7. Milstone, H., *J. Immunol.*, 1941, v42, 109.
8. Boisvert, P. L., Proc. Soc. Exp. Biol. and Med., 1942, v49, 678.
9. Kaplan, M. H., and Commission on Acute Respiratory Disease, *J. Clin. Invest.*, 1946, v25, 347.
10. Van Deventer, J. K., Proc. Soc. Exp. Biol. and Med., 1935, v33, 17.
11. Kaplan, M. H., Proc. Soc. Exp. Biol. and Med., 1946, v63, 50.
12. Mote, J. R., Massell, B. F., and Jones, T. D., *J. Immunol.*, 1939, v36, 71.
13. Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, 1934, v60, 239.
14. Commission on Acute Respiratory Diseases, *J. Clin. Invest.*, 1946, v25, 352.
15. Rantz, L. A., Boisvert, P. J., and Spink, W. W., *Science*, 1946, v103, 352.
16. Commission on Acute Respiratory Diseases and Kaplan, M. H., *Science*, 1945, v101, 120.

Received June 25, 1953. P.S.E.B.M., 1953, v83.

Relationship Between Human Seminal Fluid and the Fibrinolytic System. (20461)

KURT N. VON KAULLA* AND LANDRUM B. SHETTLES.†

From the Research Service, Third (NYU) Medical Division of Goldwater Memorial Hospital, Welfare Island, and the Department of Obstetrics and Gynecology, College of Physicians and Surgeons, Columbia University and the Sloane Hospital for Women, New York City.

Activators and inhibitors as well as precursors of plasmin, the fibrinolytic enzyme of blood, have been found in demonstrable concentrations in a variety of tissues and body fluids. Although the enzymes of semen have received a good deal of attention, they have been investigated but little from this point of view. Huggins and Neal(1) showed that human and canine semen contains a fibrin-splitting enzyme in addition to another which degrades fibrinogen, both of which presumably originate in the prostate. They stated that human seminal fluid is rich in fibrinolysin and poor in fibrinogenase. Eichenberger(2) noted decomposition of fibrin flakes by semen.

Procedure and observations. Our own experiments confirm the degradation of fibrin by seminal fluid. If one part of seminal fluid is added to 10 parts of citrated plasma and if this solution is clotted, the clot is dissolved within 24 hours. There are a number of phenomena which are in contrast to those observed with plasmin alone. The latter enzyme dissolves the clot of a fibrinogen solution consistently faster than that of human plasma with a comparable fibrinogen content, since the plasma contains appreciable quantities of antifibrinolysin. If seminal fluid is used in place of plasmin, an entirely different situation prevails. It is, however, relatively easy to find a concentration of seminal fluid (the optimum is not the same for each sample of semen) in which the plasma clot is lysed faster than the fibrinogen clot, as illustrated in Table I.

TABLE I. Fibrinolytic Action of Seminal Fluid on Fibrinogen and Plasma Clots. Fibrinogen 0.3%, 0.1 ml or plasma, 0.1 ml; aliquot seminal fluid; thrombin 200 units/ml, 0.03 ml; buffered saline ad 1.0 ml.

Seminal fluid conc. (ml)	Lysing time in min., fibrinogen exp.	Lysing time in min., plasma exp.
.5	540	300
.2	300	60
.02	180	210
.01	210	330

Since bovine fibrinogen was used as a substrate, the findings permit 2 interpretations: (a) that the fibrinolytic enzyme of human seminal fluid is species specific and therefore attacks bovine fibrin to a lesser degree or (b) that seminal fluid contains fibrinolysokinase which activates the profibrinolysin of plasma or serum. Another characteristic to be noted, which differs from purified plasmin, is that the briefest fibrinolysis times do not correspond to the highest seminal fluid concentration but to the lower ones. This could be attributed to the presence of an inhibitor. For clarification of this, a series of experiments were performed in which the following reagents were used.

1) Human semen was obtained in a clinic or was sent in for investigation. Immediately after receiving, the semen was frozen and before each experiment, which was carried out within a few days, it was centrifuged until the seminal fluid was free of spermatozoa. 2) Buffered saline: one part veronal-acetate buffer, pH 7.42, plus 2 parts of saline. 3) Fibrinogen: bovine fibrinogen, Armour, 1% solution in buffered saline. 4) Plasmin: acid activated fibrinolysin 1% of von Kaulla(6) in buffered saline. 5) Prolysin: from human serum according to Lewis and Ferguson(3), dissolved in buffered saline corresponding to one-half the original amount of serum. 6) Streptokinase-Streptodornase: Lederle. Dissolved in buffered saline and diluted to 100 units/ml. 7) Thrombin: Parke, Davis Co. Dissolved in buffered saline. 8) Human citrated plasma, 1 to 4, obtained on the day of experiment.

---

* Geigy Research Fellow.

† Markle Scholar in Medical Science.

In all of the experiments outlined below, the same arrangement was chosen for observing fibrinolysis, *viz.*, 0.06 ml of the fibrinogen solution or 0.1 ml of citrated plasma was diluted to 1 ml with buffered saline after the addition of all other reagents, and clotting was effected by the addition of 0.03 ml of thrombin solution. This system for studying fibrinolysis was satisfactory and suitable for the small amount of material available for our experiments. This latter point is to be noted especially, for with the same arrangement, other materials could be investigated such as cervical mucus or ovarian follicular fluid, whose quantity is limited to an even greater extent. The endpoint of complete fibrinolysis was taken as the moment at which the last visible bit of fibrin net disappeared. The detection of the endpoint was easier in those tubules containing air bubbles in the fibrin net. The tubules containing the preparations were not touched during the time of observation.

The following procedure was used for the fibrinolysis test of human seminal fluid: A mixture of equal parts of seminal fluid and buffered saline was incubated at 37°C with an equal volume of prolysin for varying lengths of time. At hourly intervals, 0.1 ml of the seminal fluid and prolysin solution was taken out and added to the mixture for testing fibrinolysis and the time of dissolution of fibrin noted.

It is evident from Table II that human seminal fluid contains fibrinolysokinase which activates prolysin; the maximum activation appears to be reached after 4 to 5 hours and then a loss of activity appears probably due to inactivation of the newly produced enzyme. Seminal fluid (50%) and prolysin solution in a ratio of 1:1 appeared to be the most favorable. The yields with 5 parts of 50% seminal fluid and one part of prolysin were distinctly less; those with one part of 50% seminal fluid and 5 parts of prolysin were somewhat less favorable. Prolysin alone was not activated during the periods of the experiment. In addition to fibrinolysokinase, seminal fluid also contains small amounts of profibrinolysin, since the fibrin-splitting activity of seminal fluid can be enhanced by the addition of streptokinase. These relationships are shown in Table III.

TABLE II.
Prolysin Activation by Human Seminal Fluid.

	Incubation time (hr)								
Exp.	0	1	2	3	4	5	6	7	8
	Lysing time (min.)								
1	105	50	25	10	8	8	9	14	16
2	240	120	50	25	10	9	9	11	13
3	155	70	40	25	20	13	18	18	22

TABLE III. Effect of Streptokinase on Human Seminal Fluid. Fibrinolysis test mixture + 0.1 ml streptokinase 100 units/ml + aliquot seminal fluid.

	Semen No.					
	1	2	3	4	5	6
	Lysing time (min.) of seminal fluid with streptokinase					
(.025 ml)	12	19	20	30	25	35
(.0025 ml)	44	34	40	60	63	45
	Lysing time (min.) of seminal fluid without streptokinase					
(.025 ml)	65	160	210	400	220	230

TABLE IV. Effect of Seminal Fluid on Plasmin. Buffered saline plasmin solution 1%, 0.01 ml + aliquot part of seminal fluid incubated for 20 min., then fibrinogen added. Thrombin.

	Specimen	A	B	C
		Lysing time, min.		
Addition of seminal fluid	(.2 ml)	120	350	90
	(.1 ml)	60	111	50
	(.01 ml)	8	12	19
	(.005 ml)	10	9	14
Without seminal fluid		22	22	22

In mixtures without seminal fluid streptokinase did not induce fibrinolysis.

It is thus evident that seminal fluid profibrinolysin (or a substance closely related) occurs together with fibrinolysokinase. Antifibrinolysin is also present and in smaller concentrations than in serum. Larger amounts of seminal fluid are capable of diminishing the action of plasmin while smaller concentrations show a synergistic action with plasmin. Details are given in the examples in Table IV.

Discussion. It was possible to demonstrate that as Huggins and Neal(1) have shown seminal fluid contains a fibrin-splitting enzyme. This enzyme was found in all (50) semen samples which were investigated. Its activity, although variable from sample to sample, does not seem to be influenced by the number of spermatozoa in that it was found in specimens with necro-, oligo-, and azospermia. The origin of this enzyme is not clear; perhaps it is formed by the prostate gland as Huggins and Neal have suggested. In cases of prostatic carcinoma, increased fibrinolysis was demonstrated in peripheral blood samples by Tagnon *et al.*(4). That this enzyme is added to the semen in a non-activated state and then becomes activated by fibrinolysokinase cannot be excluded. Fibrinolysokinase was demonstrated in all samples (14) which were investigated for its presence. Its activity, *i.e.*, concentration in various seminal fluid samples appears to be subject to some variation. The same is true for all samples containing material which was activated by streptokinase. Seminal fluid is the only body fluid which under normal circumstances contains free plasmin-like enzymes. Its full activity is produced by dilution. The significance of this enzyme system, especially in relation to fertility, is unknown. In any event, proteolytic enzymes frequently have been associated with certain fertility processes. Thus, Runnström(5) reached the conclusion that immediately after fertilization of metazoan eggs one or more proteolytic enzymes become activated. If one assumes that the spermatozoa are surrounded by an active fibrinolytic, seminal fluid layer, they encounter rather favorable conditions for maintaining this fibrinolytic activity in their passage to the ovum; the cervical mucus contains practically no anti-fibrinolysin, but small amounts of a material which is subject to activation by streptokinase; the uterus contains fibrinolysokinase as shown by Lewis and Ferguson(3), and also a fibrinolytic enzyme (Greenberg(7), Page *et al.*(8). Homogenized human fallopian tubal mucosa is also fibrinolytic and contains fibrinolysokinase. Follicular fluid usually contains large amounts of antifibrinolysin and also high concentrations of profibrinolysin. We shall report upon these findings in a later communication. It is thus possible that in order to perform its fertilizing function under normal circumstances the spermatozoon needs proteolytic enzymes which undergo, on its passage to the ovum, activation rather than inactivation. If these suppositions are correct, then one can draw the inference that some cases of sterility may be associated with disturbances of these enzymes.

Summary. Human seminal fluid contains in addition to fibrinolytic enzymes (whose identity with the enzymes of blood remains to be demonstrated) fibrinolysokinase, antifibrinolysin and a material which can be activated by streptokinase.

---

1. Huggins, C., and Neal, W., *J. Exp. Med.*, 1952, v76, 527; *Am. J. Physiol.*, 1943, v139, 129.
2. Eichenberger, E., *Gynaecologia*, 1946, v121, 288.
3. Lewis, J. H., and Ferguson, J. H., *J. Clin. Invest.*, 1950, v29, 1059.
4. Tagnon, H. J., Whitmore, W. F., and Shulman, N. R., *Cancer*, 1952, v5, 9.
5. Runnström, J., *Advances in Enzymology*, 1949, v9, 241.
6. von Kaulla, K. N., *Nature*, 1949, v164, 40.
7. Greenberg, E. M., *Bull. New York Acad. Med.*, 1948, v6, 397.
8. Page, E., Glendening, M. B., and Parkinson, D., *Am. J. Obstet. and Gynecol.*, 1951, v62, 1100.

Received June 25, 1953. P.S.E.B.M., 1953, v83.

Sex Differences in Effect of Radiation and Fluoracetate Poisoning on Liver Metabolism. (20462)

Margery G. Ord and L. A. Stocken. (Introduced by S. Ochoa)
From the Department of Biochemistry, University of Oxford, England.

In the course of an investigation of the effects of X-radiation on liver metabolism it was considered important to confirm the observations of Dubois, Cochran, and Doull(1), and Dubois, Cochran, and Zerwic(2) that there was a sex difference in the response given by rats to sequential exposure to X-radiation and fluoracetate poisoning. These workers claimed that no accumulation of citrate took place in the livers of male Sprague-Dawley rats injected with 3.5 mg/kg fluoracetate and killed after 3 hours; female rats showed a very marked accumulation when similarly treated. If, however, the rats were exposed to X-radiation prior to the injection of fluoracetate the males as well as the females accumulated citrate. Buffa and Peters(3) in their original experiments found little or no increase of citrate in the liver after fluoracetate injection, a result confirmed by Potter and Busch(4). Repetition of these experiments with our strain of Wistar rats suggests that in both sexes the results are very dependent on the state of nutrition of the animals; both male and female fed animals show increased liver citrate levels after fluoracetate injection, whereas starved animals show a smaller response. In the case of X-radiation we find the effect to be less dramatic than has been reported.

Materials and methods. Rats weighing 150-250 g were fed on a stock diet of rat cubes supplied by Messrs. Heygate and Sons of Northampton. In all cases liver extracts obtained from fed animals contained glycogen. When starved animals were required, food was withdrawn for 24 or 48 hours, but water was always available. The animals were irradiated from underneath in batches of 4. The characteristics of the machine were 240 kv, 15 milliamps, H.V.L. 1.2 mm Cu. The dose rate in air measured 3 cm above the floor of the cage, was 18-19 r/min. The field was uniform to ±5%.

Sodium fluoracetate (FAc) was kindly provided by Prof. R. A. Peters. It was dissolved (200 mg/100 ml 0.9% w/v NaCl) and 4 mg/kg were injected intraperitoneally into rats which were killed after 2 hours by decapitation. The livers (3-8 g) were immediately homogenized in 20 ml 10% trichloracetic acid. The residue after centrifugation was re-extracted and the combined extracts were made up to 50 ml.

Citric acid was estimated by the method of Pucher, Sherman, and Vickery(5) as modified by Buffa and Peters(3). In our hands the method, as applied to the estimation in liver, was not completely satisfactory, but no advantage was obtained by the application of other available methods to this tissue. Generally, control and experimental animals were examined in the same run so that direct comparisons could be made.

Results. The citric acid content of livers of normal animals of both sexes and the levels reached after fluoracetate injections are shown in Table I. In starved normal animals the citrate levels are about the same, but on a normal diet with food available *ad lib.*, the level in females tends to be slightly higher than in males. The sex difference in the accumulation of citrate in rat livers in response to fluoracetate previously observed by Dubois *et al.*(1,2) is less marked in our animals. In the starved animals given fluoracetate the citrate level in the liver was increased in the females, but a similar effect was found also in the males. The injection of fluoracetate into fed animals produced a very marked rise in the accumulation in female rats, but in male rats too there was a considerable increase.

Dubois *et al.* had also found that in their strain of rats irradiation caused male rats to accumulate citrate in response to fluoracetate and did not alter the level in female animals. The magnitude of the effect was proportional to their X-ray dose (200-800 r). In our experiments (Table II) with 500 r irradiation

TABLE I. Citric Acid Content of Rat Livers (μg/g Tissue).

	Starved			Fed	
	No FAc, 24 hr	With FAc 24 hr	With FAc 48 hr	No FAc	With FAc
Male	19* (14–22)†	105 (45–123)	56 (49–61)	32 (20–58)	197 (57–356)
No. animals	4	7	3	4	13
Female	27 (22–36)	116 (82–154)	—	63 (42–80)	571 (157–910)
No. animals	5	7		4	12
Castrated males	19 (18–22)	103 (62–154)	59 (47–71)	43 (32–51)	266 (110–570)
No. animals	4	7	2	4	7

* Mean. † Range.

TABLE II. Citric Acid Levels in Livers of Male Rats Exposed to 500 r X-radiation (μg/g Tissue).

	24 hr post radiation, starved 48 hr, with FAc	8 days post radiation			
		Starved 24 hr		Fed	
		No FAc	With FAc	No FAc	With FAc
	173* (123–290)†	30 (25–40)	104 (95–111)	53 (41–61)	317 (123–625)
No. animals	5	3	3	4	10

* Mean. † Range.

apparently had little effect on the normal liver citrate levels of male rats 8 days after exposure, with or without food, and in response to fluoracetate the starved liver citrate level was not different from the non-irradiated controls. If the animals were fed, however, the accumulation was greater than the control animals (Student's t test, $t = 2.38$, $P < 0.05$). Also in animals starved for 24 hours then irradiated and starved for the next 24 hours, citrate levels after fluoracetate injection were comparable to those in fed males, this presumably reflecting the increased glycogen content of the livers following irradiation (North and Nims)(6).

Discussion. Dubois *et al.*(1,2) did not regard a citrate concentration of less than 100 μg/g tissue as an accumulation, nor was any mention made of the nutritional state of the animals. It would appear therefore in the light of our experiments that the observations of Dubois *et al.* of the failure of normal male rats to accumulate citrate in their livers after fluoracetate may be principally one of degree. Sex differences in the accumulation of the acid are undeniably present, but it is also apparent that feeding the animals, as might be expected, produces a very great increase in the citrate concentration obtained in this tissue. An isolated observation of citrate accumulation in the kidneys of an injected starved female rat did not show any dependence on the nutritional state in this organ, nor have sex differences in accumulation been reported in other tissues. Moreover, no sex differences in susceptibility to fluoracetate poisoning have been reported, and it seems unlikely that differences in liver citrate content between the 2 sexes are of importance in the overall syndrome of fluoracetate poisoning in rats.

The greater accumulation in fed irradiated male rats suggests that the normal method for combatting fluoracetate poisoning in these livers has been affected by X-radiation. It is generally agreed that the interstitial cells of the testis are not affected by radiation of the order used here (Bloom)(7) so that the reaction of the livers to fluoracetate after radiation is presumably not attributable to lack of testosterone, although the effect produced is comparable to that in castrated males. The liver is usually considered to be fairly resistant to X-radiation and there are no differences in mortality between male and female rats. This suggests that the effects of X-radiation on the metabolic pathways in liver related to citrate oxidation which are specific to male rats, are immaterial to the survival of the animal.

Summary. The nutritional state of the animal is a determining factor in the accumulation of citrate in male, female and castrated male rats after fluoracetate poisoning; it is also significant when male rats are exposed to X-radiation before injection of fluoracetate.

We wish to thank Professor R. A. Peters for drawing our attention to the observations of Dubois *et al.*(1), Dr. J. F. Loutit and Mr. M. J. Corp at the M.R.C. Radio-Biological Research Unit Harwell for irradiation of the animals, and Miss W. Yates for skilled technical assistance. We are indebted to the Medical Research Council for a grant to one of us (M.G.O.).

---

1. Dubois, K. P., Cochran, K. W., and Doull, J., Proc. Soc. Exp. Biol. and Med., 1951, v76, 422.

2. Dubois, K. P., Cochran, K. W., and Zerwic, M. M., Proc. Soc. Exp. Biol. and Med., 1951, v78, 452.

3. Buffa, P., and Peters, R. A., *J. Physiol.*, 1950, v110, 488.

4. Potter, V. R., and Busch, H., *Cancer Research*, 1950, v10, 353.

5. Pucher, G. W., Sherman, C. C. and Vickery, H. B., *J. Biol. Chem.*, 1936, v113, 235.

6. North, N. and Nims, L. F., *Fed. Proc.*, 1949, v8, 119.

7. Bloom, W., *Histopathology of irradiation from internal and external sources.* 1948, McGraw-Hill Book Co., Inc., New York.

Received June 25, 1953. P.S.E.B.M., 1953, v83.

Amino Acids and Peptides in the Oviduct of the Laying Hen.*† (20463)

Halvor N. Christensen and Thomas R. Riggs.

From the Department of Biochemistry and Nutrition, Tufts College Medical School, Boston.

Inappreciable quantities of free peptides, other than certain specialized structures, appear in various tissues(1), even in tissues which are carrying out rapid synthesis of protein. This finding along with the sluggish capture(2) and utilization(3) of peptides by tissues has led us to question how extensively free peptides in general enter into protein synthesis. Anfinsen and Steinberg(4,5) however, have observed a large dilution of isotopic amino acids entering certain positions of ovalbumin, relative to other positions, during incubation of the minced oviduct of the laying hen. They have suggested that peptides present in the oviduct from the beginning of the experiment served as protein precursors, to produce the observed dilution of the labeled free amino acids. To assist in the interpretation of these results we have analyzed fresh and incubated oviducts for free and bound amino acids.

Experimental. The oviducts were excised promptly after sacrifice of the hen. In each case, except No. 4, the oviduct contained at removal an egg in the later stages of formation. The organ was divided into 2 similar samples; one part was immediately homogenized with saturated aqueous picric acid (10 ml per g) in a Waring blendor. The other portion was minced as described by Anfinsen and Steinberg(4), suspended in the medium they describe and incubated at 37°C for 2 to 4 hours. The suspension was then also treated with picric acid, 10 ml for each g of original tissue. The filtered extracts were immediately enclosed in a cellophane bag and dialyzed against measured volumes of water for 14 to 16 hours at 5°C on a rotating wheel; the dialysates were then analyzed immediately for glycine(6) and α-amino acid ("carboxyl") nitrogen at pH 2.5(7) both before and after acid hydrolysis(8).

Results. As shown in Table I, the fresh oviducts contained high levels of free amino acids. Calculation indicates that the cells were about 10 times as rich in α-amino acids in general, and in glycine, as was the plasma

---

* This investigation has been supported in part by a grant from the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

† We are indebted to Doctors Gerhard Schmidt and Maria Seraydarian for making available to us oviducts from hens used in their experiments.

TABLE I. Amino Acids and Peptides in Picric Acid Extracts of Oviduct of the Hen. Concentrations are in mM per kilo fresh tissue.

		Glycine		α-amino acids		
Exp. No.	Condition	Free	Bound	Free	Bound	Non-glutathione bound*
1	Fresh	1.87	3.19	27.9	5.2	—1.2
2	Fresh	2.10	3.24	26.7	8.1	+1.6
	Minced and incubated 2 hr	4.44	2.85	45.8	5.4	— .3
3	Fresh	1.89	3.81	27.1	6.9	— .7
	Minced and incubated 4 hr	5.58	4.16	55.5	5.4	+2.9
4	Fresh	2.27	4.81	25.4	8.4	—1.2
	Minced and incubated 4 hr	6.11	2.86	45.1	4.6	—1.1

* Calculated as (bound α-amino acids) minus 2 × (bound glycine).

of the hen. Since these levels were maintained despite the rapid synthesis of egg-white protein which presumably had continued up to the moment of the sacrifice of the bird (except in No. 4) this organ clearly is quite active in concentrating free amino acids. The calculations show that close to one-half the bound amino acid residues are due to glycine. This proportion is characteristic of glutathione, which releases one bound glycine and a second bound α-amino acid structure due to cysteine upon hydrolysis. Hence the bound amino acids in the extracts appear to be due to glutathione; whatever substantial part may not be glutathione must contain glycine as at least half of its amino acid resdues, and any "pool" of peptides of random structure must be very small.

The difference between the bound α-amino acid nitrogen and twice the bound glycine is recorded as the "non-glutathione bound α-amino acids" in the last column of Table I, and has a value close to zero, with an average value of minus 0.5 millimole per kilo of tissue. This value in one case was slightly increased after incubation, but free amino acids were by far the chief product of the net protein breakdown during incubation. Because of the high potassium content of the medium, these free amino acids would be expected to be largely extracellular.

Discussion. The possibility must be kept in mind that larger intermediates in the synthesis of protein may be precipitated with the proteins by picric acid. Mixtures of smaller peptides, however, are recovered well following picric acid deproteinization(3); therefore, the amounts of lower peptides present in a free form must be small. These results appear to set an upper limit to the amount of dilution which can be attributed to free small peptides. Unless the total amount of albumin synthesized in the experiments of Anfinsen and Steinberg is very small, it appears necessary to look for other sources of the isotope dilution they report than the free peptides present in the oviduct at the beginning of an experiment.

Summary. The oviduct of the laying hen, either fresh or after incubation in a minced state, is rich in free amino acids, but the bound, dialyzable amino acids extracted by picric acid are largely limited to glutathione or similarly glycine-rich structures.

---

1. Christensen, H. N., Rothwell, J. T., Sears, R. A., and Streicher, J. A., *J. Biol. Chem.*, 1948, v175, 101.

2. Christensen, H. N., and Rafn, M. L., *Cancer Research*, 1952, v12, 495.

3. Christensen, H. N., Lynch, E. L., and Powers, J. H., *J. Biol. Chem.*, 1946, v166, 649.

4. Anfinsen, C. B., and Steinberg, D., *J. Biol. Chem.*, 1951, v189, 739.

5. Steinberg, D., and Anfinsen, C. B., *J. Biol. Chem.*, 1952, v199, 25.

6. Christensen, H. N., Riggs, T. R., and Ray, N. E., *Anal. Chem.*, 1951, v23, 1521.

7. Hamilton, P. B., and Van Slyke, D. D., *J. Biol. Chem.*, 1943, v150, 231.

8. Christensen, H. N., and Lynch, E. L., *J. Biol. Chem.*, 1946, v166, 87.

Received June 26, 1953. P.S.E.B.M., 1953, v83.

Experimental Infection of Macacus Rhesus with *Rickettsia quintana* (Trench Fever) (20464)

HERMAN MOOSER AND FRITZ WEYER. (Introduced by Peyton Rous)
From the Hygiene-Institut der Universität, Zürich, Switzerland, and Bernhard-Nocht-Institut, Hamburg, Germany.

Of all the known rickettsiae pathogenic for man *Rickettsia quintana* exhibits the strictest host specificity. No small laboratory animal has been found susceptible to the agent of trench fever. So far all attempts at cultivation of *R. quintana* in the chick embryo and in tissue cultures have failed. The same narrow range of susceptibility to *R. quintana* obtains with respect to blood sucking arthropods. The 2 species of lice parasitizing man, *P. humanus* and *Ph. pubis,* are apparently the sole arthropods in which *R. quintana* is able to survive and multiply(1). Codeleoncini reported the successful inoculation of 2 baboons, *Papio doquerae*(2). They reacted with intermittent paroxysms of fever similar to those occurring in trench fever of man. A small percentage of the lice (*Pediculus*), which were fed on one of the baboons, became infected with extracellular rickettsiae. Codeleoncini had accidentally recovered his strain of *R. quintana* from lice which had been reared on natives in Erythrea for the preparation of Weigl's antityphus vaccine. Several cases of trench fever had occurred among them before he experimented with baboons. In a laboratory, where work is done with *R. quintana,* a small percentage of lice may become infected by feeders who never show any morbid signs. Hence the possibility that some of Codeleoncini's "clean lice" had become infected before they were transferred to the baboon cannot be excluded with certainty. Constant control of the lice of the breeding stock is indispensable in order to be able to exclude an occasional infection of a small percentage of them.

Materials and methods. Seven rhesus monkeys were inoculated intravenously, each with a suspension in nutrient broth of the intestines of 9-12 heavily infected lice. Four strains of *R. quintana* were at our disposal. Four of the monkeys were inoculated, each with the intestines of a different batch of lice infected with strain Ossijek. The remaining 3 monkeys were inoculated, each separately, with one of the 3 other strains. Strain Ossijek was isolated in Jugoslavia from cases of trench fever among prisoners of war(3). Since its isolation this strain has caused trench fever in 12 persons. Nine of the cases were due to experimental inoculation. The remaining 3 occurred spontaneously in a bacteriologist and 2 members of his family. A thirteenth person, who was inoculated twice (a Swiss, who had never been outside the country), remained entirely well. Unfortunately the blood of that person was not examined for the presence of rickettsiae. The other 3 strains of *R. quintana* were isolated in Hamburg, Germany, between 1949 and 1953. Two of these strains were obtained from lice which had been reared and fed separately on 2 healthy laboratory aides with no previous history of trench fever. According to previous conceptions these 2 strains would have to be classified as *R. pediculi,* Rocha Lima, 1917. The third strain was recovered from a person who had acted as feeder of lice during several years. He fell ill on December 20, 1952, with intermittent fever and headaches. Lice fed on him in the hospital during a whole week remained free of rickettsiae. Nevertheless, trench fever was suspected and the patient received an intensive treatment with aureomycin which was followed by prompt and lasting result with respect to clinical symptoms. However, 100% of the lice fed on him after his discharge from the hospital in January 1953 showed *R. quintana.* At present, end of May, he still infects a high percentage of the lice. These Hamburg strains have been kept going exclusively by Weigl's method since their isolation.

The feeding of *Pediculus* on monkeys is a tedious and cumbersome procedure. We therefore had recourse to Weigl's method, *viz.,* the rectal inoculation of clean lice with

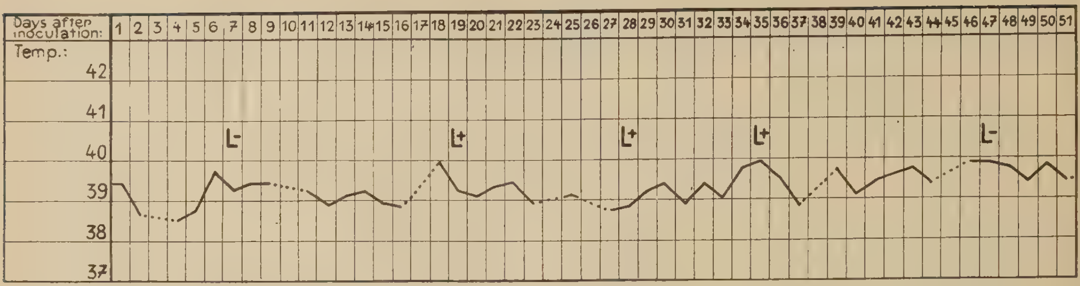


FIG. 1. Temperature chart of *M. rhesus* No. 1, inoculated on 9/29/1952, with strain Ossijek. L—, lice showed no rickettsiae. L+, lice showed rickettsiae.

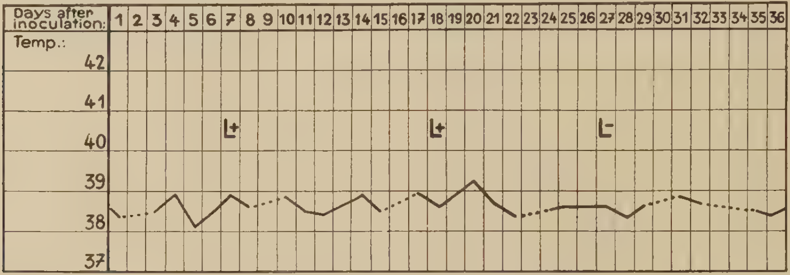


FIG. 2. Temperature chart of *M. rhesus* No. 2, inoculated on 11/21/1952, with strain Hagel I (Hamburg). L—, lice showed no rickettsiae. L+, lice showed rickettsiae.

a drop of blood drawn from the ears of the inoculated monkeys. It was diluted 1:5 with broth to prevent coagulation. Each louse received a single inoculation. In 3 instances lice were allowed a single feeding on a given monkey. In 7 louse tests performed with the monkeys' blood, the lice were inoculated through the vagina into the celom. Thereafter all lice were fed on a healthy person. The stocks of lice, from which batches were removed for the louse tests, were controlled by the daily examination of stained smears of the feces which had accumulated in the feeding boxes and by the examination of smears of the guts of frequent samples of lice taken from the stocks. The intracelomic inoculation served as an additional check of the origin of the rickettsiae which appeared in the lice which had fed on or which had been inoculated with the blood of a given monkey. *R. quintana* multiplies abundantly in the celom of the louse(4). The rickettsiae are easily demonstrated in a smear of the small drop of hemolymph which extrudes from the stump of an amputated leg. An admixture of intestinal contents with the lymph is impossible by this procedure. Rickettsiae found in the hemolymph of the lice intracelomically inoculated with the blood of one of the monkeys could therefore not derive from one of the human louse feeders. The first louse test on each monkey was performed 6-9 days after its inoculation, with one exception. The tests were repeated at intervals of about 12 days until the infectivity for lice of the blood of a given monkey had ceased. For each test 25 to 50 lice were used. All the lice which survived 5 days were examined for the presence of rickettsiae.

Course of infection in M. rhesus. None of the 7 monkeys showed definite signs of disease. Some of them seemed somewhat more restless and irritable than usual at the beginning of the blood invasion with rickettsiae, about 6-7 days after inoculation. The rectal temperature, taken every morning except Sundays, did not show any impressive alterations. In 3 monkeys there were slight peaks above the normal temperature range at irregular intervals, suggesting temperature curves of mild cases of the disease in man.

In the 4 remaining animals the temperatures remained entirely within normal limits during the whole course of observation, 42 to

100 days, respectively.

Rickettsemia. R. quintana appeared in the blood of all the 7 monkeys. The rickettsemia lasted from 10 to 82 days as estimated from the length of the interval between the first and the last positive louse test performed on each monkey. In 6 monkeys the first blood withdrawal for the louse test was performed 6, 7, 8, 8, 9, and 16 days, respectively, after the monkeys had been inoculated. All tests were positive. The blood of the 7th monkey was negative on the 7th day, positive on the 16th. The degree of the rickettsemia could be estimated approximately in each monkey from the percentage of lice which became positive in each consecutive test. In the lice which had been inoculated rectally, the proportion of positive lice reached from 39 to 100%, respectively. Materially the same percentages of positive lice were obtained with intracelomic inoculation. Of the 3 batches of lice which were fed, each on a different monkey, one batch consisted of freshly hatched larvae, the other 2 consisted of adult females. Ten per cent of the larvae became infected with *R. quintana,* whereas of the females 79 and 100%, respectively, became infected. The last positive louse test performed on each monkey scored 4 to 24% of infected lice. Considering the fact that the lice received either a single intrarectal or intracelomic injection of blood diluted 1:5, or were allowed only a single feeding, the high percentage of positive takes indicated a considerable degree of rickettsemia in all our monkeys. After a single feeding on a human case of trench fever, the percentage of lice showing rickettsiae, in our experience, may be much lower than that observed in these 7 *rhesus* monkeys.

Summary. Rhesus monkeys were successfully inoculated with one or another of 4 strains of *R. quintana.* Several of the animals showed a long-lasting rickettsemia. As is the case in trench fever of man, the infection in some of the monkeys remained subclinical. There was no correlation between clinical manifestations and the degree of rickettsemia. The *rhesus* monkey is a satisfactory animal for the experimental study of trench fever.

---

1. Weyer, F., *Z. Tropen. Parasitol.,* 1952, v3, 302.
2. Codeleoncini, E., *Boll. Soc. Ital. Med. Ig. Trop,* 1946, v6, 129.
3. Mooser, H., Leeman, A., Chao, S. H., and Gubler, H. U., *Schweiz, Z. Path. Bakt.,* 1948, v11, 513.
4. Weyer, F., *Z. Tropen. Parasitol.,* 1950, v2, 40.

Received June 26, 1953. P.S.E.B.M., 1953, v83.

CH Hemagglutinogen in Primates.* (20465)

DONALD C. A. BUTTS (Introduced by R. T. Hill)
(With the technical assistance of Doren E. Bergstresser)
From the Department of Tropical Disease Research, Division of Research and Industry, University of Miami and the Veterans Administration Hospital, Coral Gables, Fla.

Since the Ch factor was first reported(1), our efforts have been directed toward the development of high titer anti-Ch serum devoid of any heterospecific agglutinins and to extend our observations to individuals of all blood groups rather than limiting our studies to individuals of blood group O, as was previously done.

Preparation of anti-Ch serum. Prior to starting the immunization, the sera from young, male rabbits were tested for their normal heterospecific titer using OMN Rh-positive washed human erythrocytes for the determination. In no case were rabbits employed which showed the presence of species agglutinins in a titer exceeding 1-32. Following such screening, immunization with washed chimpanzee cells was started. Two male

---

* Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the author are the results of his own study and do not necessarily reflect the opinion of the Veterans Administration.

TABLE I. Protocol Illustrating Absorption of Anti-CH Serum by Means of Human Cell Stroma and Packed Washed Human Cells.

Date	Absorption No.	Cell suspension*	Dilution of serum after addition of cell suspension (figure below each dilution indicates degree of agglutination)													
			1–2	1–4	1–8	1–16	1–32	1–64	1–128	1–256	1–512	1–1024	1–2048	1–4096	1–8192	1–16384
5–13	Unabsorbed	Group A	4	4	4	4	4	4	4	4	3+	3	—	—	—	—
		B	4	4	4	4	4	4	4	4	3+	2	—	—	—	—
		O	4	4	4	4	4	4	4	4	3+	1	—	—	—	—
		Chimpanzee†	4	4	4	4	4	4	4	4	4	4	4	2	1+	—
Serum absorbed with equivalent vol of stroma from groups A and B human erythrocytes for 3 hr at 30°C, followed by 15 hr at 5°C																
5–14	1	Group A	4	4	4	4	4	4	4	3+	1	—	—	—	—	—
		B	4	4	4	4	4	4	4	4	2	—	—	—	—	—
		O	4	4	4	4	4	4	4	3+	1+	—	—	—	—	—
		Chimpanzee†	4	4	4	4	4	4	4	4	4	4	4	2	1	—
Serum reabsorbed with equivalent vol of fresh stroma for 2 hr at 30°C with constant agitation																
5–14	2	Group A	4	4	4	4	4	4	3+	3	1	—	—	—	—	—
		B	4	4	4	4	4	4	4	3	1+	—	—	—	—	—
		O	4	4	4	4	4	4	4	3+	1+	—	—	—	—	—
		Chimpanzee†	4	4	4	4	4	4	4	4	4	3+	1	—	—	—
Serum reabsorbed with 2 equivalent vol of fresh stroma for 3 hr at 37°C with frequent agitation																
5–15	3	Group A	4	4	4	4	4	4	3	2+	1	—	—	—	—	—
		B	4	4	4	4	4	4	3+	3	1	—	—	—	—	—
		O	4	4	4	4	4	4	4	3+	1	—	—	—	—	—
		Chimpanzee†	4	4	4	4	4	4	4	4	3	2+	1+	—	—	—
Serum reabsorbed with equal vol of packed washed ABMN, Rh_0-positive human erythrocytes for 2 hr at 33°C with frequent agitation																
5–20	4	Group A	4	4	4	4	4	2+	1	—	—	—	—	—	—	—
		B	4	4	4	4	4	4	2+	1+	—	—	—	—	—	—
		O	4	4	4	4	4	4	3+	3	—	—	—	—	—	—
		Chimpanzee†	4	4	4	4	4	4	4	4	3	2	1	—	—	—
Serum reabsorbed with half vol of packed washed ABMN, Rh_0-positive human erythrocytes for 3 hr at 30°C without agitation																
5–20	5	Group A	4	4	4	3+	3	2+	—	—	—	—	—	—	—	—
		B	4	4	4	4	4	3+	2+	1+	—	—	—	—	—	—
		O	4	4	4	4	3+	3	—	—	—	—	—	—	—	—
		Chimpanzee†	4	4	4	4	4	4	4	4	3	2	1	—	—	—
Serum reabsorbed with half vol of packed washed OMN, Rh_0-positive human erythrocytes for 2 hr at 30°C with frequent agitation																
5–21	6	Group A	4	4	4	3+	2+	1	—	—	—	—	—	—	—	—
		B	4	4	4	2+	1+	1	—	—	—	—	—	—	—	—
		O	4	4	4	4	3	2	—	—	—	—	—	—	—	—
		Chimpanzee†	4	4	4	4	4	4	4	3+	3	1	—	—	—	—

chimpanzees (*Pan troglodytes verus*) both OM Rh_0hr' were employed. These were selected from the group recently described by the author(2).

Immunization was started by the intravenous injection of a 20% sterile saline suspension of washed (3 times) chimpanzee cells. The usual amount initially injected was 2 ml. In general, these were followed in one week by a second intravenous injection of a 20% cell suspension from the same chimpanzees. Seven days following the second injection, blood was withdrawn from the rabbits and the serum, after inactivation, was checked for titer against OMN Rh-positive human cells. Generally, we found that the titer was now 1-256. Similar injections at weekly intervals were usually given intraperitoneally and continued until a titer of 1-1024 to 1-2048 was obtained when checked separately against a 2% suspension of washed A, B, O, and AB human cells. When such titers were reached, blood from all rabbits was withdrawn from the heart. This was allowed to clot and clear serum obtained by centrifugation. If any hemolysis was present, the serum was rejected.

Absorption technic. After inactivation at 56°C for 30 minutes, the sera were pooled prior to absorption. In order to reduce hemolysis which usually developed after repeated absorptions of high titer sera with packed human erythrocytes, our initial step was to absorb the serum with given volumes of stroma† from group A and B, Rh_0-positive human erythrocytes. Each volume of stroma represents an equivalent volume of mixed A and B cells. Table I shows the results obtained throughout 3 absorptions with varying amounts of stroma and varying conditions of absorption. It should be noted that the third stroma absorption failed to alter the titer of the serum and only slightly modified the degree of agglutination of cells in any of the serum dilutions employed. Further absorptions were carried out with washed packed human cells, as described in Table I,

TABLE I (continued).

Date	Absorption No.	Cell suspension*	Dilution of serum after addition of cell suspension (figure below each dilution indicates degree of agglutination) 1–2	1–4	1–8	1–16	1–32	1–64	1–128	1–256	1–512	1–1024	1–2048	1–4096	1–8192	1–16384
Serum reabsorbed with half vol of packed washed OMN, Rh_0-positive human erythrocytes (Negro donor) for 2 hr at 30°C with constant agitation																
5–22	7	Group A	4	4	4	3+	1	—	—	—	—	—	—	—	—	—
		B	4	4	4	3+	1+	—	—	—	—	—	—	—	—	—
		O	4	4	4	3+	1+	—	—	—	—	—	—	—	—	—
		Chimpanzee†	4	4	4	4	4	4	4	3	1	—	—	—	—	—
Serum reabsorbed with half vol of packed washed OMN, Rh_0-positive human erythrocytes for 2 hr at 31°C with frequent agitation																
5–28	8	Group A	3+	2	1	—	—	—	—	—	—	—	—	—	—	—
		B	3+	2	1	—	—	—	—	—	—	—	—	—	—	—
		O	3+	2+	1	—	—	—	—	—	—	—	—	—	—	—
		Chimpanzee†	4	4	4	4	4	4	3+	3	1	—	—	—	—	—

* Mixture of washed cells from 2 or more group A, B or O humans. All mixtures contained cells of M and N types and all were Rh_0-positive.

† Washed cell suspensions.

---

† The stroma used in these absorptions was generously donated by the Medical Research Foundation of Dade County, Miami, Fla. The preparation of this has, to date, not been published.

until no further reduction was noted in the reaction with chimpanzee cells. We consider the values, after a total of 8 absorptions, represent the titer of the specific Ch antibody.

It was possible, by an additional absorption with OMN Rh-positive human cells, to remove the antibody so that the serum failed to react with human cells of any blood group. However, absolutely no alteration resulted in the degree of agglutination of chimpanzee cells in any serum dilution and the end-point remained constant (1-512). After the titration of absorbed serum, the undiluted serum was filtered through a Seitz filter and to this filtrate was added Merthiolate (1-10,000). Tests showed that such treatment failed to alter further the titer of the serum.

Slide test. Using the serum absorbed to the point where agglutination of human cells still occurred in a titer of 1-8, a screening test was performed. For this purpose, 3 or 4 drops of finger blood were placed on a slide to which one drop of the anti-Ch serum was added. These were mixed by means of a wooden applicator to cover an area of approximately two-thirds that of the slide. The slides were then placed on a viewing box at a temperature of 42°-45°C and tilted back and forth for a period of 2 minutes. The interval after which agglutination was first apparent was reported as the "appearance time" and the relative degree of agglutination was recorded at the end of 2 minutes.

Results of slide tests. Before starting studies on human bloods, we tested the blood of several chimpanzees, spider monkeys (*Ateles geoffroyi*), rabbits and mice. Two male chimpanzees (*Pan troglodytes verus*) and one female pygmy chimpanzee (*Pan paniscus*) were tested. One of the males was OM Rh$_0$hr′, the other male and the female were AM Rh$_0$hr′. All 3 chimpanzees' blood reacted with the anti-Ch serum in 5 seconds and all showed a complete agglutination reaction with this serum within 2 minutes.

The bloods of spider monkeys, rabbits and mice failed to react with the anti-Ch serum within the 2-minute period.

We first tested 57 Negroes by this method. Of these, 55 (96.5%) were Ch-positive and 2 (3.5%) were found to be Ch-negative. We

TABLE II. Slide and Tube Agglutination Reactions of Chimpanzee and Human Erythrocytes with Anti-CH Serum, with Special Reference to Family of C.W. (No. 3-7).

					Slide test		Tube test									
							Serum dilutions after addition of cell suspensions									
No.	Description	Sex	Race	Blood group	App. time	React. time after 2 min.	1-2	1-4	1-8	1-16	1-32	1-64	1-128	1-256	1-512	1-1024
	Chimpanzee	♂		AM†	5 sec.	++++	4	4	4	4	4	4	3+	3	1	—
	"	♂		OM†	5	++++	4	4	4	4	4	4	3+	2+	1	—
	"	♀		AM†	5	++++	4	4	4	4	4	4	3+	3	1	—
1	M.B.	♀	N	BM†	18	++	3+	2+	1+	—	—	—				
2	G.F.	♂	N	OM†	—	—	2+	1+	—	—	—	—				
3	C.W.	♂	N	ON†	—	—	1+	1	—	—	—	—				
4	" sister	♀	N	NR	NR	NR	1	—	—	—	—	—				
5	" "	♀	N	NR	NR	NR	2	2	—	—	—	—				
6	" father	♂	N	NR	NR	NR	3+	3+	3	2+	1	—				
7	" mother	♀	N	NR	NR	NR	—	—	—	—	—	—				
8	A.B.	♂	W	OMN†	15 sec.	++	3+	3+	2	—	—	—				
9	J.C.	♂	W	AN†	—	—	1	—	—	—	—	—				

* Pygmy chimpanzee.

† Rh-positive when tested with anti-Rh$_0$ (anti-D) serum.

NR = Not run.

were forced to limit our studies on Whites to 35 as our available supply of absorbed serum was practically depleted. Of these, 34 (97.1%) were Ch-positive and one (2.9%) was Ch-negative. The ABO group and the MN and Rh types in both Whites and Negroes were distributed within the limits of recognized percentages.

Tube test. To study further the individuals whose blood gave a negative slide test, we prepared serial dilutions of anti-Ch serum with saline. To each dilution, an equal volume of a 2% washed cell suspension of the individual's blood was added, the contents agitated and then centrifuged at 2,000 r.p.m. for 3 minutes. The degree of agglutination was then determined by examination over a viewing box.

Results of tube tests. Tube tests with chimpanzee blood and anti-Ch serum confirmed results of slide tests. Regardless of species, sex or blood group, the degree of agglutination in each serum dilution is almost identical and the end-point (1-512) is the same in each.

We selected at random from the slide tests, one White and one Negro who showed a minimal appearance time and a maximal agglutination after 2 minutes. Reference to Table II shows their reaction in the tube test as compared to the chimpanzee and those individuals who were negative on the slide test. Since the families of Nos. 2 and 9 (Table II) were not available, our studies in the genetics of this factor were limited to the family of No. 3. The blood of his mother, father and 2 sisters were checked and the results are shown in Table II. From these data, we assume the father to be homozygous Ch-positive while the mother was homozygous Ch-negative; whereas the children were heterozygous Ch-positive, thereby strongly suggesting that the Ch agglutinogen is a transmitted Mendelian dominant.

Summary. In our first paper describing this factor, the serum was diluted 1-10 prior to a single absorption with washed packed OMN Rh-positive human erythrocytes. Our present serum has been absorbed, undiluted, under varying conditions, 3 times with cell stroma from group A and B human erythrocytes and 5 times with washed packed human erythrocytes of groups A, B, and O. It is of interest that all chimpanzees of both group A and group O and of both sexes, reacted in almost an identical manner when tested by either the slide or tube test method. One of these was a pygmy chimpanzee (*Pan paniscus*). After 6 absorptions (Table I) no alteration in the titer occurred with chimpanzee cells. Although our studies are not complete, we have presented the following facts: That an anti-chimpanzee (anti-Ch) serum of high antibody titer has been developed by repeated injections of washed chimpanzee cells into rabbits. That the great majority of both Whites and Negroes contain the Ch agglutinogen, in varying degrees, in or on their erythrocytes. Individuals do exist, however, whose erythrocytes completely lack this hemagglutinogen. That, as in all other human blood factors, the Ch factor is an apparently inherited characteristic, transmitted as a Mendelian dominant and that it is not associated with any of the recognized hemagglutinogens of man. We are continuing studies on a relatively large number of individuals of different races in an effort to further determine the racial distribution of this factor and to enable us to work out, in a more comprehensive manner, the genetics of our problem.

---

1. Butts, D. C. A., *Am. J. Trop. Med.*, 1950, v30, 663.

2. ———, *Am. J. Phys. Anthrop.*, 1953, n.s., 11, 215.

Received June 29, 1953. P.S.E.B.M., 1953, v83.

Further Modification of the Mouse Adapted Type III Poliomyelitis Virus.

(20466)

C. P. Li and Morris Schaeffer.

From the Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Montgomery, Ala.

Recently, Li and Habel(1) reported the adaptation of poliomyelitis Type III (Leon) virus to mice, employing the intraspinal route (2). This has been confirmed by Casals *et al.*(3). The virus was pathogenic only after intraspinal inoculation and could be recovered from the spinal cord, but not from the brain. Although the incubation period was relatively short, usually 4-10 days, it was inconstant. In addition, the low virulence of the virus limited its usefulness in laboratory and field studies. For this reason attempts were made to increase its virulence for mice by a number of procedures, including the use of cortisone, varying host ages and strains, and exposure of mice to cold before and after inoculation. None of these was successful. With consecutive mouse passage, however, despite some fluctuation, the virulence gradually increased. This was particularly obvious after 69 passages, when the incubation period was sharply reduced.

Materials and methods. The mouse adapted Type III (Leon) virus, originally reported by Li and Habel(1), was brought to this laboratory in its 54th passage. Consecutive passage in mice was then made by the intraspinal route with 10% or 20% cord suspension derived from paralyzed mice and inoculated into 4- to 6-week-old CFW mice.

Hyperimmune serum was prepared in monkeys with adjuvants following the procedure of Ward *et al.*(4) against Type I (Brunhilde), Type II (Lansing), and Type III (Leon) monkey propagated stock viruses. The technic for tissue culture utilized in this study was described previously(5) and the technic of neutralization tests is described elsewhere(6).

Experimental. A number of methods were employed in an effort to increase the virulence of the virus, but it appears that this occurred spontaneously with consecutive mouse passages, and notably after the 69th passage. Two obvious changes in the behavior of the virus took place. These were an increase of the titer of the virus in the mouse cord and a shortening and fixing of the incubation period (Tables I and II).

TABLE I. Comparison of Titration of Earlier and Later Passage Mouse-Adapted Leon Virus.

Passage	Dilution of virus			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Pool of mouse passage 20, 21, 22	4/8*	2/17	0/8	
Pool of mouse passage 72, 73, 74	11/12	6/11	5/11	0/12

* No. mice paralyzed/No. inoculated.

In the early passages, the inoculation of a 20% mouse cord suspension did not paralyze all the mice. When such a suspension was mixed with serum and .02 ml of the mixture was inoculated intraspinally into mice, the PD$_{50}$ was less than one log. It was not suitable, therefore, for neutralization tests. In the later passages, the titer increase to $10^{-2.5}$ to $10^{-3.0}$ so that the inoculation of a mixture of 20% virus suspension and serum represents 30-100 PD$_{50}$, a dose more adequate for reliable and reproducible results. In the early passages the incubation period was not constant and varied from 4 to 10 days or longer, while in the later passages the incubation period not only was shortened but also became more constant. An occasional shortening of incubation period to 2 days was first observed on the 69th passage.

In carrying out neutralization tests on about 110 human serum specimens with this virus, entirely clear-cut results were obtained (6). Out of 587 mice inoculated in these tests, 435 became paralyzed and of these more than 80% were paralyzed within 7 days after inoculation. This characteristic of the modified virus enhances its usefulness in laboratory and field studies.

Besides the 2 main changes noted, the virus can now occasionally infect mice by the intra

TABLE II. Comparison of Incubation Period of Earlier and Later Passage Mouse-Adapted Leon Virus.

Passage	Suspension, %	Paralysis	Incubation period	Avg incub. period (days)
55	20	17/22*	3^{2}†, 4^{2}, 5^{4}, 6^{4}, 8^{4}, 9^{1}	5.8
56	20	13/18	4^{4}, 5^{4}, 6^{2}, 7^{1}, 10^{2}	5.7
69	10	7/9	2^{1}, 3^{2}, 4^{1}, 5^{1}, 6^{1}, 8^{1}	4.1
70	10	11/11	2^{1}, 3^{3}, 4^{7}	3.5
74	10	8/8	3^{8}	3.0

* No. mice paralyzed/No. inoculated. † 2 mice paralyzed on 3rd day.

TABLE III. Neutralization Tests with Modified Mouse-Adapted Leon Virus against Monkey Immune Sera.

	Serum of monkey immune to types							
	I Brunhilde		II Lansing		III Monkey Leon		Normal monkey serum	
	Undil.	1/10	Undil.	1/10	Undil.	1/10	Undil.	1/10
Paralysis*	8/9	9/10	10/10	9/9	0/10	3/10	9/10	9/9

* No. mice paralyzed/No. inoculated, observed 11 days.

cerebral route. This was also recently observed by Habel(7). The serologic specificity of the virus has not been altered. It is still specifically neutralized by Leon immune serum but not by other antipoliomyelitic sera (Table III).

The original Leon virus had a high degree of virulence for the monkey. With passage through mice the virulence appears to be decreasing. A 20% mouse cord suspension of the 73rd passage was inoculated into 2 adult rhesus monkeys by the intracerebral route. One of them became paralyzed on the 14th day, showing typical histopathological lesions in the cord. The other remained asymptomatic for an observation period of over 45 days when it was sacrificed and showed no histopathologic lesions in the CNS. Virus from mouse cord in the 73rd mouse passage was cultivated in monkey testis tissue culture. After 6 serial passages in this tissue culture, the tissue culture fluid was inoculated intracerebrally as well as intranasally to 2 rhesus monkeys. One remained well for 30 days; the other showed slight weakness of the left leg in the second week after inoculation but recovered rapidly. Histopathologic examination of this monkey, sacrificed on the 30th day after inoculation, revealed the typical lesions of poliomyelitis in recovery phase.

A rather interesting observation involving virulence was made in the course of study with this virus. When the virus from infected mouse cord suspension was transferred to monkey testicular tissue culture, cellular degeneration occurred in 4-9 days. However, when the pooled fluid harvested successively within a period of 3 weeks or ground tissue fragments from such infected cultures were inoculated into mice intraspinally, the virulence was lost entirely or was greatly reduced (Table IV). This occurrence is in distinct contrast with the behavior of the mouse-adapted Type I (Mahoney) virus which when grown in monkey testicular tissue culture infects mice quite readily(8). Further studies with respect to the virulence of this virus are in progress.

Summary. A further modification of the mouse-adapted Leon virus is described. With successive mouse passages the virulence for

TABLE IV. Virulence Loss of Mouse-Adapted Leon Virus after One Tissue Culture Passage.

Mouse passage No.	Inoculated into:		Paralysis	Avg incubation period
P55	mice		6/10	7.5
P55	t.c.*	mice	1/9	7.0
P56	t.c.*	"	0/10	0
P72	mice		6/8	3
P72	t.c.†	"	2/8	7
P73	t.c.‡	"	0/10	0
P79	mice		64/72	3
P79	t.c.*	"	4/40	5.2

* Undiluted fluid of tissue culture inoculated to mice.
† Tissue fragments inoculated to mice.
‡ Six consecutive passages in tissue culture.

mice has spontaneously increased and the incubation period has become shorter and more constant. Passage from mice to tissue culture results in loss or marked reduction of virulence for mice. This modified virus can be employed quite satisfactorily for serum neutralization tests and makes available an additional field and laboratory tool for poliomyelitis research.

---

1. Li, C. P., and Habel, Karl, PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 233.

2. Habel, Karl, and Li, C. P., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 357.

3. Casals, J., Olitsky, P. K., and Brown, L. V., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 731.

4. Ward, Robert, Rader, D., Lipton, M. M., and Freund, Jules, PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 536.

5. Li, C. P., and Schaeffer, M., *Science*, 1953, v118, 107.

6. ———, in preparation.

7. Habel, Karl. Personal communication.

8. Li, C. P., and Schaeffer, M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 477.

Received June 29, 1953. P.S.E.B.M., 1953, v83.

Effect of Cold and Restraint on Blood and Liver Non-Protein Sulfhydryl Compounds. (20467)

R. G. BARTLETT AND U. D. REGISTER. (Introduced by D. W. Visser)

From the Departments of Physiology and Biochemistry, School of Medicine, College of Medical Evangelists, Loma Linda, Calif.

Beck and Linkenheimer(1) have reported a drop in concentration of liver non-protein sulfhydryl (glutathione) in mice exposed to cold and indicated that this fall in concentration was not due to the fall in body temperature which occurred(2). In the present studies 2 groups of rats (restricted and unrestricted) were exposed to cold. Liver and blood ergothioneine (ESH), glutathione (GSH), and total non-protein sulfhydryl (TSH) concentrations were compared with values for control animals.

Methods and materials. Healthy adult Sprague-Dawley rats were used: The 21 males (250-300 g) and 21 females (180-250 g) were divided into 3 groups each: 7 controls exposed at 22°C, 7 unrestricted exposed at 0°C, and 7 restricted exposed at 0°C. The control rats were housed in regular laboratory cages until time of sacrifice while the latter 2 groups were placed, for periods varying between 2 and 4 hours, in a refrigerator set to maintain a temperature of 0°C ± 2°C. The duration of exposure was determined by the time necessary for the rectal temperatures of the restricted animals to fall to between 15 and 25°C. Restraint was produced by means of a loose fitting wire mesh cylinder. All the animals were stunned with a blow on the head, blood obtained with a cardiac puncture and liver excised immediately and frozen with dry ice. ESH was determined by a modified method of Hunter(3); GSH by a modification of the method of Grunert and Phillips(4) and TSH by amperometric titration using a modification of the method of Benesch and Benesch(5). Details of the modifications will be published(6).

Results. As shown in Table I, no significant change in GSH or TSH concentration in the blood of rats was produced by restraint or cold. There was a slight drop in ESH. Similar treatment produced no change in ESH concentrations of the liver. However, there was a significant drop in the concentration of GSH and TSH in the livers of the restricted animals as compared to the control animals and to those which were maintained unrestricted in the cold. The fall in concentration of both GSH and TSH of the liver was more marked in the females than in the males under the conditions of the present experiment.

Discussion. The data showing a decrease in liver TSH in animals maintained in the cold are in agreement with the work of Beck and Linkenheimer(1,2). They measured only

TABLE I. Effect of Cold and Restraint on Rat Blood and Liver Ergothioneine, Glutathione and Total Non-protein Sulfhydryl Concentrations.

		Ergothioneine, μM %	Glutathione, μM %	Total non-protein sulfhydryl, μM %
		Liver		
Controls (22°C)	♂	89.3 ± 2.3[†]	765 ± 19	955 ± 16
Body temp. (38-40°C)	♀		722 ± 21	821 ± 37
Unrestricted (0°C)	♂	96.6 ± 4.5	729 ± 44	863 ± 31
Body temp. (37-39°C)	♀		667 ± 14	
Restricted (0°C)	♂	83 ± 3.8	612 ± 27	742 ± 32
Body temp. (15-25°C)	♀		480 ± 17	581 ± 37
		Whole blood		
*Controls (22°C)	♂	15.9 ± 0.3	122 ± 6	154 ± 5
*Unrestricted (0°C)	♂	13.0 ± 0.3	99 ± 5	161 ± 9
*Restricted (0°C)	♂	11.3 ± 0.9	108 ± 8	161 ± 10

* No significant difference in the hematocrit (avg value = 47).

[†] Stand. error of the mean.

a 1-2°C drop in body temperature of unrestricted mice(1). This was essentially the same as the fall in rectal temperatures occurring in the unrestricted rats in the present series. It has been demonstrated by Bartlett *et al.*(7,8) that the stress of restraint, as used here, is at least partially emotional in nature. By the use of such stress body temperatures of small mammals are made to fall precipitously(7,8). Whether the marked decrease in liver GSH and TSH was due to the lowered body temperature or to the emotional stress is not known. Studies are under way to clarify this point. Beck and Linkenheimer reported(2) that the fall in body temperatures was not responsible for the fall in liver TSH in mice. Mortensen(9) has suggested that blood GSH may be maintained at the expense of liver GSH. The present studies seem to indicate a similar relationship. It is noted in Table I that the drop in TSH in the liver is primarily due to a drop in the concentration of GSH.

It has been suggested that one of the functions of GSH is to maintain enzymes in their active sulfhydryl form(10). Since a number of enzymes in carbohydrate and lipid metabolism depend upon glutathione and other sulfhydryl compounds such as coenzyme A for their activity, any lowering of sulfhydryl compounds in the blood or liver may interfere with carbohydrate and lipid metabolism. Perhaps the low levels of GSH coupled with the low liver glycogen levels (qualitatively noted) resulted in a decreased ability to supply energy which could have been a factor in the loss of body temperature.

Summary. Adult male and female Sprague-Dawley rats were exposed to cold. Half of those so exposed were stressed by use of restraining cages. Ergothioneine (ESH), glutathione (GSH), and total non-protein sulfhydryl compounds (TSH) of blood and liver were determined. There was a slight fall in blood ESH and no change in blood GSH or TSH. Simultaneous exposure to cold and restraint produced a significant fall in liver GSH and TSH, but not in ESH.

---

1. Beck, L. V., and Linkenheimer, W. H., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 291.

2. Linkenheimer, W. H., and Beck, L. V., *Fed. Proc.*, 1953, v12, 88.

3. Hunter, G., *Canad. J. Res. Sect. E.*, 1949, v27, 230.

4. Grunert, R. R., and Phillips, P. H., *Arch. Biochem.*, 1951, v30, 217.

5. Benesch, R. E., and Benesch, R., *Arch. Biochem.*, 1950, v28, 43.

6. Register, U. D., to be published.

7. Bartlett, R. G., Helmendach, R. H., and Bohr, V. C., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 4.

8. Bartlett, R. G., Bohr, V. C., and Helmendach, R. H., to be published.

9. Mortensen, R. A., *J. Biol. Chem.*, in press.

10. Barron, E. S. G., *Advances in Enzymol.*, 1951, v11, 201.

Received June 29, 1953. P.S.E.B.M., 1953, v83.

Effect of Repeated Zirconium Citrate Injections on Distribution of Plutonium in the Rat.* (20468)

PATRICIA L. HACKETT. (Introduced by Austin M. Brues)

From the Biology Section, Radiological Sciences Department, General Electric Co., Richland, Wash.

Schubert has shown that early administration of zirconium citrate to rats injected with plutonium results in a marked decrease in the skeletal deposition of this element(1). An additional experiment by the same worker demonstrated that the skeletal content of plutonium was nearly independent of the amount of zirconium injected (with a minimum amount of zirconium between 5.1 and 12.3 mg/kg) and of the number of doses of zirconium given, although the author believed that prolonged treatment with zirconium citrate would probably effect actual removal of plutonium from bone(2).

Most of the previous information(1-3) on the administration of zirconium citrate to animals receiving plutonium has been derived from relatively short-term experiments (3-6 days). The purpose of this report is to present some preliminary data on the effect of divided doses of zirconium citrate over a longer period of time.

Methods. Sixteen Sprague-Dawley male rats with weights averaging 290 g were divided into 4 groups of 4 rats each. Rats in the zirconium toxicity control group received 15 injections (3 per week), intraperitoneally, of 4 ml of a "zirconium citrate" solution prepared according to Schubert(1) and containing 12.8 mg zirconium per ml. All rats in the remaining 3 groups received a single intraperitoneal injection of 27.3 μg of Pu (IV) in 2 ml of 2% citric acid solution adjusted to pH 5.6. One of these plutonium-injected groups did not receive zirconium injections. Zirconium citrate was administered to the second group as described above for the toxicity control group, the first injection being given 30 minutes after the animals had received plutonium. The last group received zirconium citrate injections commencing 32 days after the plutonium injection.

It was planned to sacrifice all animals 71 days after the plutonium injection, but 3 animals were killed early because of a respiratory infection and otitis media. Corresponding animals from other groups were sacrificed at the same time for comparison.

Upon sacrifice, the viscera were removed and the remainder of the animal autoclaved for 30 minutes at 15 lb steam pressure to facilitate separation of the skeleton. The loss of plutonium from the bone when this procedure was used was found to be insignificant. Skeleton, liver, spleen, and residual carcass were ashed with concentrated nitric acid and the resulting acid solution was pipetted onto planchets for counting(4).

Results and discussion. Fig. 1 shows the average weight curves for the 4 groups of rats. Two rats which exhibited large weight losses due to respiratory infection were excluded from the averages. It is evident that the zirconium citrate injections were accompanied by significant weight loss, and the weight gains followed termination of these injections. Histological study of the livers of the zirconium toxicity control rats showed no evidence of damage. These results are similar to those obtained by McClinton and Schubert(5).

Results of plutonium analysis are shown in Table I. Animals 1 and 9 should probably not be considered in comparisons, since they lost about 30% of their initial weight before sacrifice.

The previously reported effect of early zirconium citrate treatment on skeletal deposition of plutonium is clearly confirmed. A 6-fold reduction in skeletal deposition is seen in rats 5, 6, and 7, and about a 12-fold reduction in rat 8; however, the plutonium content of all the various tissues from rat 8 is uniformly low.

Perhaps the most interesting finding of this study was the very marked increase in retention of plutonium in the soft tissues (residual

---

* Based on work performed under contract W-31-109-Eng.-52 for the Atomic Energy Commission.

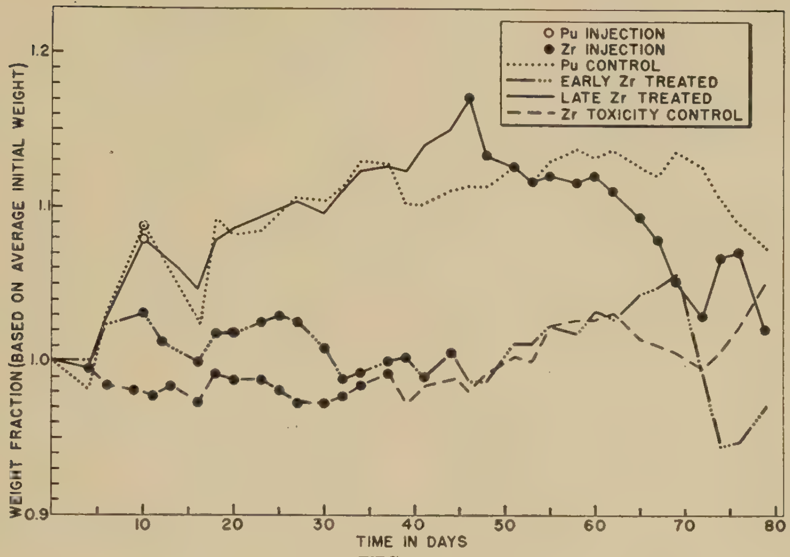


FIG. 1.

carcass) which resulted from the early zirconium citrate treatment. The 2 rats sacrificed during the zirconium citrate treatment (5 and 6) retained 6 to 9 times as much plutonium in the residual carcass as did the controls. Even a month after termination of the zirconium citrate treatment, rats 7 and 8 had 2 to 5 times as much plutonium in the residual carcass as did the controls. In preventing the skeletal deposition of plutonium, the long-term administration of zirconium citrate had also the positive effect of fixing the plutonium in the soft tissues. These findings are not in complete accord with later work from this laboratory(6); however, previous workers(7) have suggested that the eventual tissue distribution of the zirconium colloidal aggregates on which the circulating plutonium is adsorbed is a function of the colloid's particle size. It may be that minor variations in the procedure for preparing the zirconium citrate solution would result in a change of particle size.

Because of the enhanced soft tissue retention of plutonium, the effectiveness of the zirconium citrate treatment in decreasing the

TABLE I. Retention and Distribution of Plutonium.

Group	Animal No.	Time of sacrifice (days after Pu inj.)	No. Zr injections	Total mg Zr injected	% of injected plutonium retained				
					Liver	Spleen	Skeleton	Residual carcass	Total
Pu control	1*	20	—	—	3.4	.29	46	6.9	57
	2†	62	—	—	1.7	.39	66	4.7	73
	3	71	—	—	3.1	.45	62	5.0	70
	4	71	—	—	4.1	.44	70	4.6	79
Early Zr treated	5‡	20	9	463	7.3	.52	11	44	63
	6§	27	12	617	4.0	.22	11	31	46
	7	71	15	771	4.0	.53	11	23	39
	8	71	15	771	2.1	.14	5.1	10	19
Late Zr treated	9‖	56	9	463	.89	.11	29	5.7	36
	10¶	62	11	565	2.3	.47	63	4.6	70
	11	71	15	771	4.0	.25	58	7.6	70
	12	71	15	771	—	.18	53	10	—

* Died of respiratory infection after large wt loss. † Died suddenly of respiratory infection. ‡ Sacrificed early for comparison with control #1. § Sacrificed after development of respiratory infection. ‖ Sacrificed after large wt loss for comparison with rat #5. ¶ Sacrificed early for comparison with control #2.

total plutonium body burden is much less dramatic than is its effect on skeletal deposition. By a comparison of the total injected dose retained in the Pu controls and the early zirconium treated animals it appears, however, that this plutonium retained in soft tissue may be lost from the body at a considerably faster rate than plutonium deposited in the skeleton. It also seems evident that the soft tissue plutonium is not redistributed to the skeleton after termination of the zirconium citrate treatment (rats 7 and 8).

The late zirconium citrate treatment had perhaps a slight effect both in decreasing the amount of skeletally deposited plutonium and in increasing the amount in the residual carcass. Further studies would be required to establish the significance of these effects. No differences of particular significance were observed in the plutonium content of livers and spleens of rats in the different groups.

Summary. Serial zirconium citrate injections during the month immediately following the administration of plutonium were found to effect a 6-fold reduction in the skeletal deposition of plutonium and to increase the retention of plutonium in the soft tissues. Therapy with zirconium, commencing a month after the plutonium injection, was found to have little, if any, effect on the distribution of plutonium.

Sincere appreciation is extended Dr. Joseph Katz, Mrs. Willa D. Oakley and the Radiochemistry Laboratory for their assistance during the course of this work.

---

1. Schubert, J., *J. Lab. Clin. Med.*, 1949, v34, 313.
2. Schubert, J., and White, M. R., *J. Biol. Chem.*, 1950, v184, 191.
3. Kawin, B., Copp, D. H., and Hamilton, J. G., Univ. of Calif. Radiation Lab. Unclassified Document No. 812.
4. Schubert, J., Finkel, M. P., White, M. R., and Hirsch, G. M., *J. Biol. Chem.*, 1950, v182, 635.
5. McClinton, L. T., and Schubert, J., *J. Pharm. Exp. Therap.*, 1948, v94, 1.
6. Katz, J., Manuscript in preparation.
7. White, M. R., and Schubert, J., *J. Pharm. Exp. Therap.*, 1952, v104, 317.

Received June 30, 1953. P.S.E.B.M., 1953, v83.

Use of Baby Chicks for Isolation of Leptospires. (20469)

WARREN G. HOAG, WILLIAM S. GOCHENOUR, JR., AND ROBERT H. YAGER.
From the Veterinary Division, Army Medical Service Graduate School, Washington, D. C.

Guinea pigs(1), embryonated hens' eggs (2), Syrian golden hamsters(3), and laboratory white mice(4) have been employed in the isolation of leptospires from blood, tissues, and other body fluids. Their employment for this purpose has been limited by factors of cost, availability, and facilities appropriate for their maintenance. The successful cultivation of leptospires in embryonated hens' eggs suggested the possible utilization of baby chicks for recovery of these organisms.

Methods. Two-day-old chicks were inoculated intraperitoneally with 0.5 ml of 5-day-old culture of *L. pomona*, S91,(5) in Fletcher's semi-solid medium(6). Subsequent culture generations were lethal for 20 g golden hamsters. Heart's blood cultures were made in both Schuffner's modification of Verwoort's medium(6) and Fletcher's semi-solid medium on the 4th, 7th, 8th, 9th, 10th, 11th, 14th, and 21st days post-inoculation.

Groups of chicks were sacrificed on the 9th, 11th, 14th, and 21st days post-inoculation. Kidney and liver emulsions in 0.85% NaCl solution were cultured separately on both leptospiral mediums. These cultures were incubated at 30°C for 28 days and examined by darkground microscopy at 7-day intervals.

Serums were obtained by clot-squeeze separation from blood drawn by cardiac puncture on the 9th, 11th, 14th, and 21st days post-inoculation. These serums were examined for presence of leptospiral antibodies by the

TABLE I. Results of Heart's Blood Cultures.

Chick No.	Days post-inoculation 4	7	8	9	10	11	14	21
1	+	+	ND	+*				
2	+	+	ND	+*				
3	+	+	ND	+*				
4	+	ND	+	ND	ND	+*		
5	+	ND	+	ND	ND	+*		
6	+	ND	+	ND	ND	—*		
7	+	ND	ND	ND	+	ND	+*	
8	+	ND	ND	ND	—	ND	—*	
9	+	ND	ND	ND	+	ND	+*	
10	ND	ND	ND	ND	ND	ND	ND	+*
11	ND	ND	ND	ND	ND	ND	ND	+*
12	ND	ND	ND	ND	ND	ND	ND	—*
13	ND	ND	ND	ND	ND	ND	ND	+*

* Animals sacrificed.
Key: + = Leptospires recovered. — = No leptospires recovered. ND = Not done.

TABLE II. Results of Culture of Tissue Emulsion.

Days post-inoculation	Liver Pos.	Liver Neg.	Kidney Pos.	Kidney Neg.
9	1	2	1	2
11	2	1	2	1
14	2	1	2	1
21	0	4	0	4

TABLE III. Agglutinating Antibody Response.

Days post-inoculation	Titer against *L. pomona* Less than 1:100	1:100	Greater than 1:100
9	3	0	0
11*	2	0	0
14*	1	1	0
21	1	2	1 (6400)

* Serum samples available on only 2 chicks.

agglutination-lysis technic(6).

Results. The results of heart's blood cultures are shown in Table I. The results of cultures of tissue emulsions are shown in Table II.

It is apparent from the above that leptospiremia persisted in all chicks examined through the 9th day post-inoculation. It is significant that leptospires could not consistently be demonstrated in cultures of either kidney or liver emulsions.

Table III lists the results of serological examination of the post-inoculation serums of these chicks. With but one exception, serologically demonstrable antibodies were at extremely low levels or absent.

No clinical symptoms of illness were apparent in any of the chicks. All appeared healthy at the time of sacrifice and no gross changes were evidenced at autopsy.

Comments. Previous work on experimental leptospirosis in chickens resulted in few recoveries of inoculated organisms(7). However, older birds were employed in these studies. The uniform development of leptospiremia following inoculation with infective material suggests the possible utilization of baby chicks in the recovery of leptospires from material unsuitable for direct cultural methods. It is, therefore, suggested that such materials be inoculated intraperitoneally and that heart's blood cultures in suitable leptospiral mediums be made on the 4th and 7th days post-inoculation. Replicate cultures should be made in order to detect leptospires which may be present only in limited numbers. Neither clinical illness nor serological response can be used as criteria of infection in chicks of this age. It is noteworthy that only an inconstant and transient renal infection was observed. The possible role of fowls in the epidemiology of leptospirosis requires further investigation.

Summary. Two-day-old chicks uniformly developed leptospiremia persisting for at least 5 days following intraperitoneal inoculation of leptospires. No clinically detected illness resulted from this infection nor was there constant significant serologically demonstrable antibody response. Employment of chicks for recovery of leptospires from materials contaminated with other microorganisms and hence unsuitable for direct culture is suggested.

---

1. Van Thiel, P. H., *The Leptospiroses,* Universitaire Pers Leiden, 1948.

2. Morrow, G., Syverton, J. T., Stiles, W. W., and Berry, G. P., *Science,* 1938, v88, 384.

3. Randall, R., and Cooper, H., *Science,* 1944, v100, 133.

4. Larson C. L., *Public Health Rep.,* 1941, v56, 1546.

5. Gochenour, W. S., Jr., Johnston, R. V., Yager, R. H., and Gochenour, W. S., *Am. J. Vet. Res.,* 1952, v13, 158.

6. Gochenour, W. S., Jr., Yager, R. H., Smadel, J. E., Wetmore, P. W., and Hightower, J. A., *Am. J. Pub. Health,* 1953, v43, 405.

7. Bernkopf, H., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 148.

Received June 30, 1953. P.S.E.B.M., 1953, v83.

Mumps Infectivity Studies in Hamsters. (20470)

M. M. Burr and F. P. Nagler.

From the Virus Section, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

The transmission of mumps to experimental animals other than monkeys(1), cats(2), guinea pigs(3), suckling mice(4), and suckling hamsters(5) has not proved successful in the past. Attempts to infect adult hamsters with this agent by various routes so far have met with failure(6).

In the course of producing specific antisera in adult hamsters, it was observed that these animals, after intranasal instillation of the virus, yielded sera with high levels of complement fixing antibodies against the soluble and viral mumps antigens. These findings led to the investigation of the pathogenicity of mumps for hamsters and to attempts at serial passage of the virus in hamster lungs. The serological and tissue responses in these animals to the intranasal inoculation of both egg-adapted and current epidemic strains of mumps virus will be described in this report, together with results of attempts to adapt the virus to lung tissues of adult hamsters.

Materials and methods. The Enders strain of mumps, which had been received from the Children's Hospital, Philadelphia, in 1949, and had undergone 20 allantoic passages in chick embryos in this laboratory, was used as the standard strain throughout these experiments.

Golden hamsters of 7-10 weeks of age were inoculated intranasally under ether anesthesia with 0.3 ml amounts of allantoic fluid containing $10^{8.5}$ EID_{50}/ml of mumps virus. A batch of 3-4 animals was anesthetized and sacrificed by exsanguination from the brachial artery each day for a period of 38 days, commencing 24 hours after inoculation and the sera of each batch were pooled. Lungs, spleens, and parotid glands of each batch were similarly pooled and 25% suspensions of each pool of organs were prepared by grinding with quartz in a mortar in physiological saline. The suspensions were centrifuged at 1500 r.p.m. for 5 minutes and the supernates were removed, shell-frozen and stored at —32°C until used. The tissue suspensions, to which were added 1000 units of penicillin and 1000 μg of dihydrostreptomycin per ml, were titrated in the amniotic sac of 9-day-old chick embryos and the amount of virus present in these suspensions was estimated by testing the amniotic fluids for presence of specific hemagglutinins, using Salks' method(7). The 50% infectivity titer (EID_{50}) was calculated by Reed and Muench's method(8).

Attempts at virus isolation from 4 throat washings of patients acutely ill with mumps were carried out in parallel series, one part of the washings being inoculated intranasally into hamsters, another part being inoculated amniotically into developing chick embryos.

Mumps antisera used in the complement fixation (C.F.) and hemagglutination inhibition (H.I.) tests of this work were prepared in hamsters by a single intranasal inoculation of 0.3 ml of allantoic fluid containing $10^{8.5}$ EID_{50}/ml of mumps virus. The animals were bled 14 days after inoculation and their sera were pooled. Antisera against the newly isolated strains were prepared in a similar manner. Details of serological procedures used in this work are described earlier(9).

Results. The serological responses of hamsters to a single intranasal inoculation of the egg-adapted Enders strain of mumps virus are summarized in Table I. The tests were carried out on pools of serum collected daily from 3-4 animals from the first day after inoculation. For simplification of tabulation, the results of these tests have been grouped together over 4-day periods and their mean values are recorded in Table I. The C.F. titers are expressed as reciprocal values of the original dilution of serum giving complete fixation in the presence of 2 units of complement. Hemagglutination titers are expressed as reciprocal values of the highest final dilution of serum causing complete inhibition of 4 hemagglutinating units of a mumps virus suspension. It can be seen from Table I that

TABLE I. Serological Response of Hamsters to Experimental Infection with Mumps Virus.

Animals No.	Days after infection	Mean values of C.F. titer with Soluble antigen	Viral antigen	Haemagglutination-inhibition titer
24	1- 6	0	0	0
16	7-10	88	56	160
16	11-14	128	112	300
16	15-18	112	128	360
12	19-22	64	85	213
12	23-26	43	64	240
8	27-30	32	64	160
12	31-34	37	43	133
16	35-38	22	40	160

TABLE II. Mumps Virus Titers in Lungs of Hamsters after Inoculation with $10^{7.8}$ EID_{50} of Infected Allantoic Fluid.

Days after inoculation	Virus recovered per g of lung*
1	4.53
2	3.46
3	3.01
4	3.82
5	2.09
6	.99
7	$<.57$
8	$<.57$

* Figures represent logs of reciprocals of the EID_{50} based on haemagglutination titers.

TABLE III. Recovery of Mumps Virus from Hamster Lungs on Serial Lung Passage.

Passage No.	Virus recovered per g of lung* Exp. series 1	Exp. series 2
1	3.66	4.4
2	3.4	4.1
3	4.05	4.8
4	3.7	5.2
5	4.59	4.4
6	4.79	4.3
7	5.06	4.1
8	3.8	3.3
9	3.5	3.3
10	4.4	2.3
11	0	0

* Figures represent logs of reciprocals of the EID_{50} based on haemagglutination titers.

C.F. antibodies against the soluble antigen do not appear in the serum of hamsters before the 7th day following inoculation. They reach a maximum level between the 11th and 14th day after inoculation. The antibodies against the viral antigen appear also at the 7th day after inoculation and they reach a maximum level between the 15th and 18th day. The decrease in antibody levels to the soluble antigen precedes the decrease in antibody levels to the viral antigen by approximately 5 days. Antibodies against both antigens, however, are still present in significant titers as late as the 38th day after experimental infection. Anti-hemagglutinins also appear on the 7th day, reach a maximum level between the 15th and 18th day and decrease in a manner similar to the C.F. titers. They are still present in significant titer on the 38th day after infection.

Results of attempts at isolation of virus from the lung tissues after a single intranasal inoculation of allantoic fluid containing $10^{8.5}$ EID_{50}/ml of mumps virus are presented in Table II. The results indicate that the virus was present in the lungs in significant titer up to the 4th day after inoculation. It decreased considerably from the 4th to the 6th day and could not be recovered from the 7th day on.

Results of attempts to transmit the virus by serial passage through lungs of hamsters are given in Table III. In the first series of experiments, transmission of the virus was successful in 10 consecutive passages with significant amounts of virus demonstrable in the lung tissues of animals sacrificed 3 days after inoculation. No virus could be detected in the lungs at the 11th-13th passages and no antibody response could be shown in surviving animals of these groups. A second series of experiments in which hamsters were sacrificed on the 3rd, 4th, and 5th day after inoculation gave very similar results to those of the first series of experiments (Table III). Judging by these results, the decrease in virus content of the lung tissues on passage apparently did not follow a simple dilution factor. Hence, attempts are being made at present to adapt the virus to these tissues by alternating passages through hamster lungs and the amniotic sac of chick embryos.

Attempts at isolation of mumps virus from throat washings by parallel passages through chick embryos and hamsters were carried out successfully in 4 instances. In one of these attempts, the virus was carried through 12 continuous passages in hamster lungs, where it attained a titer of $10^{4.3}$ EID_{50}/g of lung during the first 2 passages. However, as in the case of the egg-adapted Enders strain, the virus was lost in the following passages and no antibodies developed in the serum of surviving animals of these groups. With the 3 other attempts, virus was carried only through a limited number of hamster passages, with titers attained similar to that above. It was found that virus isolated from throat washings by intranasal inoculation into hamsters was readily adaptable to chick embryos.

Histopathology. Examination of animals sacrificed on the 4th and 5th day after inoculation with allantoic suspensions of virus revealed macroscopic focal lesions in the lungs. The histopathological examination of these lungs showed edema of the bronchial walls and fibrous tissues of larger blood vessels, with an infiltration of lymphocytes and large mononuclear cells into these areas. The alveolar walls over wide areas were much thickened due to congestion of alveolar capillaries and an infiltration of mononuclear cells. The alveolar spaces in such areas were empty or contained coagulated fluid of high protein content. In some areas there was a sparse accumulation of mononuclear phagocytes within the alveoli and occasional red blood cells were seen in these regions. The picture represented that of an interstitial pneumonitis.

Lungs of control animals, which had been inoculated similarly with a mixture of equal volumes of mumps virus and undiluted specific antiserum, showed complete absence of evidence of interstitial or alveolar exudate and their microscopic appearance was essentially that of normal lung tissues. Lungs of hamsters inoculated with a mixture of mumps virus and Influenza A (PR8) antiserum represented a microscopic picture very much the same as that of lungs of the animals inoculated with the virus suspension only.

Spleens and parotid glands of these hamsters appeared normal on macroscopic and microscopic examination. This finding confirmed results of several unsuccessful attempts at isolation of virus from portions of these organs. Histological examination of lung sections of hamsters inoculated with the 4th lung passage of the freshly isolated strain of mumps virus revealed round cell infiltration of the smaller bronchial walls and surrounding interstitial tissue. The picture represented that of a bronchitis with a focal interstitial pneumonitis.

Summary. Adult hamsters inoculated intranasally with the egg-adapted Enders strain of mumps virus produced complement fixing antibodies against both the soluble and viral antigens. These antibodies were present in measurable amounts from the 7th day after inoculation until the 38th day when the experiment was terminated. Antibodies against specific hemagglutinins also appeared on the 7th day, reached a maximum level and decreased in a similar manner to the complement fixing antibodies. The production of antibodies against the soluble antigen was considered to be indicative of virus multiplication in the tissues of the animals. Multiplication was then demonstrated by recovery of the virus after serial passage through hamster lungs. Mumps virus has been isolated from hamster lungs following intranasal inoculation of throat washings from 4 patients with epidemic parotitis. Serial passage in hamsters carried out with one of these strains gave results similar to those obtained using the egg-adapted Enders strain. Histopathological examination of lungs of hamsters inoculated either with the egg-adapted Enders strain or with a lung passage of a freshly isolated strain of mumps revealed the presence of bronchitis and interstitial pneumonitis. Specific antiserum when mixed with the standard virus suspension and similarly inoculated prevented such pathological changes, while non-specific serum had no such effect.

The authors are indebted to Dr. D. Magner and Miss G. Hein of the Canadian Tumour Registry for

the examination and the preparation of the tissue sections.

---

1. Johnson, Claud D., and Goodpasture, Ernest W., *J. Exp. Med.*, 1934, v59, 1.

2. Wollstein, M., *J. Exp. Med.*, 1916, v23, 353.

3. Bolin, Vern S., and Leymaster, Glen R., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 7.

4. Kilham, L., and Murphy, H. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 495.

5. Kilham, L., and Overman, John R., *J. Immunol.*, 1953, v70, 147.

6. Cabasso, Victor, Ruegsegger, James M., Looser, George K., and Cox, Herald, R., *J. Lab. and Clin. Med.*, 1950, v35, 771.

7. Salk, H. A., *J. Immunol.*, 1944, v49, 87.

8. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

9. Nagler, F. P., Burr, M. M., and Gillen, A. L., *Can. J. Pub. Hlth.*, 1951, v42, 367.

Received July 3, 1953. P.S.E.B.M., 1953, v83.

Reactive Sulfhydryl and Disulfide Groups of Protyrosinase and Tyrosinase.[*]

(20471)

JOSEPH HALL BODINE AND LOREN D. CARLSON.[†]

From the Zoology Laboratory, State University of Iowa, Iowa City, Iowa.

The methods of conversion of protyrosinase, the inactive form of tyrosinase, to tyrosinase have been the object of extensive studies(1). In general, it may be said that amphipathic anions, carbamates, and temperatures between 60° and 70°C for 5 minutes will bring about this change. The object of this and subsequent studies is an attempt to ascertain what physical or chemical changes occur in such a conversion.

Materials and methods. Protyrosinase was obtained from diapause grasshopper eggs. These eggs were triturated with a mortar and pestle and diluted with 0.9% NaCl to a volume of 10 cc per g of eggs. This was centrifuged and the lipoidal layer removed. The remaining supernatant was recentrifuged, decanted and 1 g of KH_2PO_4 per 250 cc of supernatant added. After standing 2 hours at 0°C, this was centrifuged and the supernatant decanted. Enough $(NH_4)_2SO_4$ was added to make the supernatant 19.3% saturated (12% solution). Two hours later the precipitate was centrifuged away and the supernatant brought to 31.1% saturation with $(NH_4)_2SO_4$ (18% solution). After 2 hours at 0°C, this was centrifuged, the supernatant discarded and the precipitate resuspended in a volume of 0.9% NaCl equal to one-half that of the solution centrifuged. After one hour this was centrifuged, the precipitate discarded and the supernatant brought to 31.1% saturation with $(NH_4)_2SO_4$. After 2 hours, this was centrifuged and the precipitate resuspended in a volume of NaCl equal to that used in the previous step. This was dialyzed against 3 changes of NaCl for at least 24 hours. The resulting preparation showed no tyrosinase activity and contained on an average 0.5 mg of nondialyzable material per cc, which in turn had in it 0.21% copper and 14% nitrogen.

A modification of the methods employed by Mirsky and Anson(2) was used to determine the reactive sulfhydryl and disulfide groups. The limitations of this method have been adequately discussed by these authors. In the determination of the sulfhydryl groups, one cc of proenzyme or enzyme plus 2 cc of 0.9% NaCl plus 0.1 cc of phosphate buffer (pH 7.5) plus 0.1 cc of 0.1 M $K_3Fe(CN)_6$ were allowed to stand at 35°C for one hour. At the end of this period, the protein was precipitated with 0.1 cc of 0.05 N H_2SO_4 and 3.3 cc of a saturated solution of $(NH_4)_2SO_4$, filtered out and one cc of Folin and Malmros(3) ferric sulfate solution added to 3 cc of the supernatant. This was allowed to

---

[*] Aided by grant from The National Institutes of Health.

[†] Now in Department of Physiology, University of Washington, Seattle.

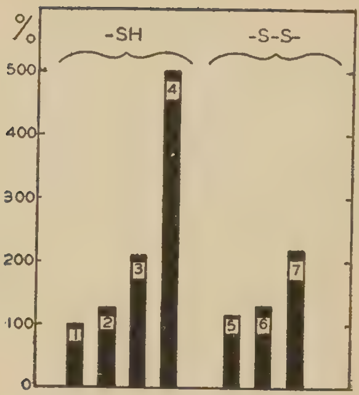


FIG. 1. Percentage amounts of sulfhydryl groups (columns 1 to 4) and disulfide groups (columns 5 to 7). Column 1, 5: the native proenzyme; column 2: the enzyme produced by Aerosol OT; column 3: the enzyme produced by exposure to 65°C for 10 min.; column 4, 7: the enzyme denatured by exposure to 100°C for 10 min.; column 6: the enzyme produced by sodium dodecyl sulfate (dialyzed away after the activation of the proenzyme).

stand 30 minutes at 35°C and the color intensity determined in a photoelectric colorimeter designed and constructed similarly to that described by Evelyn and Cipriani(4). A red filter (Corning No. 241) was used. The disulfides were determined by reduction with thioglycolate and then testing for the increase in sulfhydryl groups. Three to 5 cc of the proenzyme or enzyme solution were used with 2.5 cc of neutralized thioglycolate (app 0.7 M) and 0.5 cc of phosphate buffer (pH 7.5). The protein was precipitated with $(NH_4)_2SO_4$ and washed 4 times. A control underwent similar treatment with the exception that a NaCl solution was added in place of the thioglycolate. The precipitated protein was tested for sulfhydryl groups.

The colorimeter was standardized with known quantities of $Na_4Fe(CN)_6$. With the possible exception of very low concentrations of the ferrocyanide, the ammeter readings were linear with increasing concentrations between 0 and 8 x 10^{-7} mols of ferrocyanide added and were accurate to within 2%.

Results. The native proenzyme reduced 1 x 10^{-7} mols of ferricyanide per cc of proenzyme. This is expressed as 100% in column 1, Fig. 1. When the enzyme was prepared using Aerosol OT, a slight increase in reactive sulfhydryls occurred (Col. 2, Fig. 1), but analysis of the data of 10 different determinations showed this to be nonsignificant. Activation of the proenzyme at 65°C for 10 minutes, resulted in a significant increase in —SH groups (Col. 3, Fig. 1). This is undoubtedly due to the enzyme that is concomitantly denatured. In the data of Bodine and Allen(5), about 28% of the enzyme is destroyed by exposure to 65°C for 10 minutes. The increase in —SH noted here is approximately 27% of the amount of —SH liberated upon complete denaturation by heat (Col. 4, Fig. 1). This increase upon heat denaturation is in agreement with the findings on other proteins(6). The amount of reactive disulfide present is slightly greater than the —SH as measured by these methods (Col. 5, Fig. 1) and no significant increase occurs upon activation (Col. 6, Fig. 1). The change from proenzyme to enzyme was brought about in this case by sodium dodecyl sulfate which was then dialyzed away to facilitate precipitations. Heat denaturation doubles the number of reactive —S—S— (Col. 7, Fig. 1). It is then evident that no significant increase in —SH nor —S—S— groups is occasioned by the change from protyrosinase to tyrosinase.

It next seemed of interest to ascertain whether either the oxidation of the —SH by ferricyanide or the reduction of —S—S— with thioglycolate influences the activity of the enzyme. Both substances delayed the oxidation of the substrate by reaction with the intermediary products rather than by a primary effect on the enzyme. This is evident in Fig. 2, which shows the effects of the addition of small amounts of $K_3Fe(CN)_6$ to the reaction mixture of tyrosinase and substrate. The ferricyanide delays the initiation of the oxygen uptake and also the appearance of the red color in the reaction mixture. When the time to reach 100 μl oxygen uptake is plotted against the concentration of ferricyanide, the points, save that at zero concentration, fall on a straight line (inset). Extrapolation of this straight line gives evidence that some of the ferricyanide has been reduced to ferrocyanide by reaction with the proenzyme. It is interesting to predict that if 1 x 10^{-6} moles of ferricyanide delayed the initiation of oxygen uptake or color formation 30 minutes, then 1 x

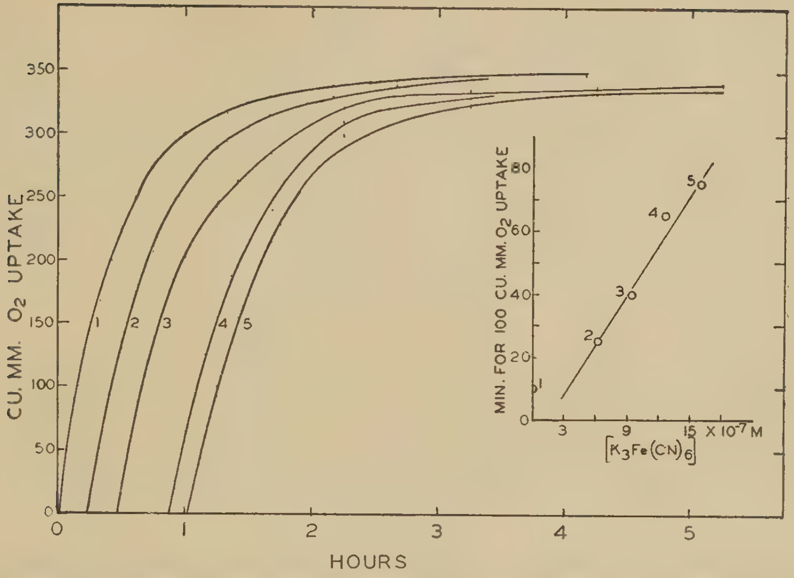


FIG. 2. Effect of potassium ferricyanide on activity of tyrosinase on tyramine-HCl. Abscissa, time in hr; ordinate, mm³ of oxygen. Concentrations of ferricyanide used may be read from the corresponding numbers on the inset. Inset: time to reach an oxygen uptake of 100 μl (ordinate) plotted against the moles of $K_3Fe(CN)_6$ added to the reaction mixture (abscissa).

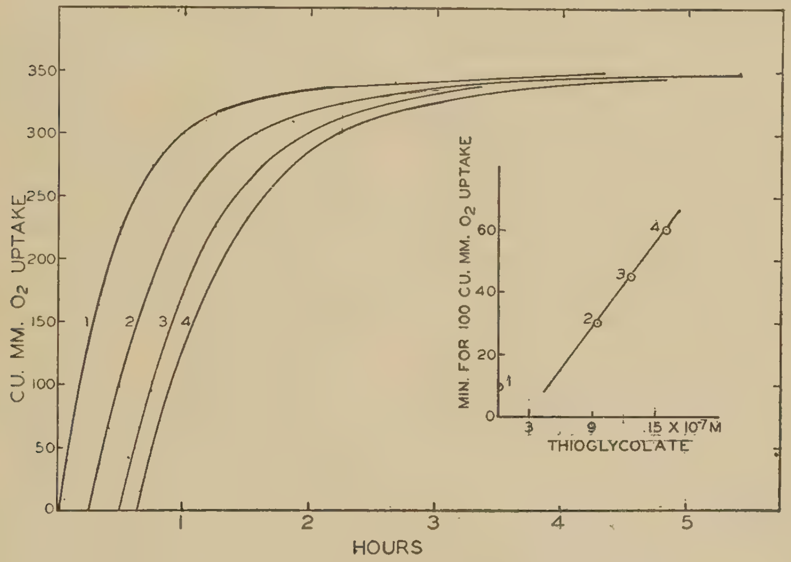


FIG. 3. A plot similar to Fig. 2, but for thioglycolate.

10^{-3} moles would delay the reaction for more than 20 days. Sodium ferrocyanide had no effect on the enzyme activity. In Fig. 3, similar data are shown using thioglycolate. Note on the inset that the straight line extrapolated indicates a greater number of —S—S— groups, this being in agreement with the colorimetric determinations.

Summary and conclusions. It seems evident that no change in sulfhydryl nor disulfide groups occurs upon activation of protyrosinase and further that the sulfhydryl and disulfide groups do not seem essential for enzyme activity. Any apparent inhibition caused by oxidation of the sulfhydryl groups or the reduction of the disulfide groups is due to a reaction of the oxidant or reductant with the intermediary products of the oxidation of tyramine-HCl. If the production of tyrosinase is occasioned by the denaturation of an inhibitor, an increase in —SH groups seems probable; this, however, is not the case.

---

1. Allen, T. H., and Bodine, J. H., *Proc. Nat. Aacd. Sc.*, 1941, v27, 269.

2. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, 1934, v18, 307.

3. Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, v83, 115.

4. Evelyn, K. A., and Cipriani, A. J., *J. Biol. Chem.*, 1937, v117, 365.

5. Bodine, J. H., and Allen, T. H., *J. Cell. and Comp. Physiol.*, 1938, v12, 71.

6. Mirsky, A. E., *Cold Spring Harbor Symposia*, 1941, v9, 278.

Received July 3, 1953. P.S.E.B.M., 1953, v83.

Inhibition of Utilization of Aspartic Acid for Growth of *Leuconostoc mesenteroides* P-60. (20472)

EUGENE ROBERTS AND PATRICIA F. HUNTER.
From the Wernse Laboratory of Cancer Research, Washington University School of Medicine, St. Louis, Mo.

γ-L-Glutamylhydrazide was an effective inhibitor of growth of 2 strains of streptococci(1) and of *L. arabinosus*(2) under conditions in which small amounts of glutamine produced a marked acceleration of growth. In both instances it was found that β-aspartylhydrazide was a weaker inhibitor than the glutamyl derivative. α-DL-Methylglutamic acid was also found to be an inhibitor of the utilization of D- and L-glutamic and α-ketoglutaric acids by *L. arabinosus*(2,3), while α-DL-methylaspartic acid and α-DL-methylasparagine were ineffective. It was of interest to determine whether the aspartic acid analogues could inhibit the utilization of aspartic acid under suitable circumstances.

Materials and methods, compounds used. L-Aspartic acid was employed in all experiments. γ-L-glutamylhydrazide (GGH), β-DL-aspartylhydrazide (BAH), α-DL-methylglutamic acid (AMG), α-DL-methylaspartic acid (AMA), and α-DL-methylasparagine (AMAS) were kindly given to us by Dr. Karl Pfister of Merck and Co., Inc. The procedure of Hac and Snell(4) was employed for microbiological assay of L-aspartic acid with *L. mesenteroides* P-60, and growth was determined turbidimetrically and the results expressed in optical density units (O.D.). Solutions of all of the substances tested for inhibition were sterilized by Seitz filtration. The final volume of the assay medium was 1.5 ml per tube. The basal medium and the test samples were added to the assay tubes with the rapid automatic dispenser of Cannon.

Experimental results. In Fig. 1A is seen a typical standard curve obtained after 75 hours of incubation at 37°C, showing the graded response of the bacteria to increasing amounts of L-aspartic acid. An experiment was performed simultaneously in which incubations were made with 60 γ of L-aspartic acid per tube in the presence of 1-5 μM of the ana-

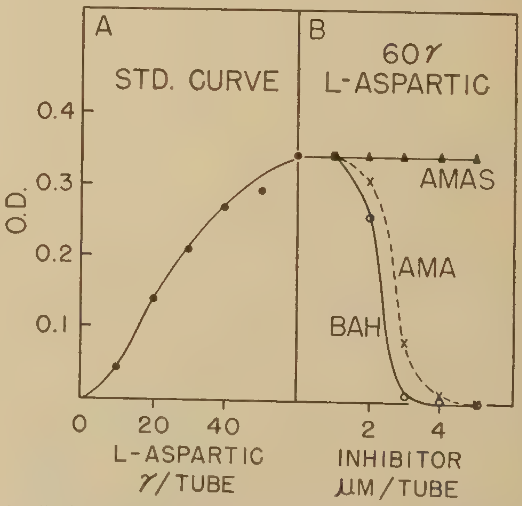


FIG. 1. Influence of varying amounts of aspartic acid analogues on growth on 60 γ of L-aspartic acid. BAH, β-DL-aspartylhydrazide; AMA, α-DL-methylaspartic acid; AMAS, α-DL-methylasparagine.

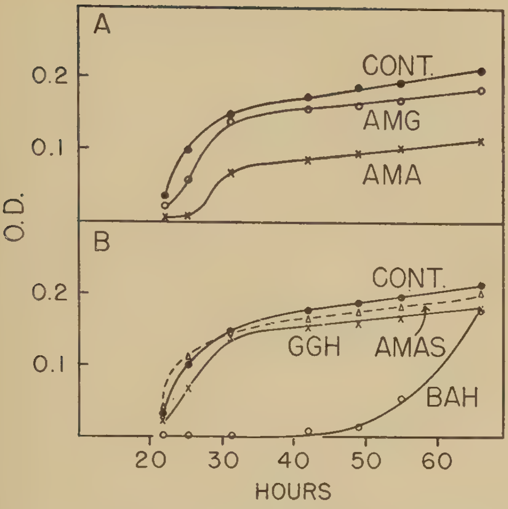


FIG. 2. Comparison of inhibition by glutamic acid and aspartic acid analogues. GGH, γ-L-glutamylhydrazide; AMG, α-DL-methylglutamic acid.

logues (Fig. 1B). Both BAH and AMA were found to be relatively effective inhibitors of growth, giving 50% inhibition at molar ratios of inhibitor to metabolite of 4.9:1 and 5.9:1, respectively, while AMAS was ineffective. In further experiments it was shown that the complete inhibition of growth produced by 5 μM of GGH in the presence of 60 γ of L-aspartic acid was not reversed by the presence of an additional 10-50 γ of L-aspartic acid or 20 γ of asparagine. When L-aspartic acid was added to similarly constituted incubation mixtures containing 5 μM of AMA, no growth occurred until the total content of L-aspartic acid was increased to 90 and 100 γ per tube, under which conditions there occurred 31 and 42%, respectively, of the growth with 60 γ of L-aspartic acid in the absence of inhibitors. Although the degree of inhibition given by a particular concentration of analogue varied somewhat from one experiment to another, the relative efficacy remained the same.

The results in Fig. 2 show the time-course of growth on 60 γ of L-aspartic acid in the absence of inhibitor and in the presence of 2 μM per tube of the 5 test substances. It is seen that the aspartic acid analogues, AMA and BAH, are much more effective than their glutamic acid counterparts, AMG and GGH. The latter 2 substances were, at most, only slightly inhibitory. The presence of asparagine up to 16 γ per tube did not alter the results obtained, in keeping with the relative ineffectiveness of asparagine in this system(4).

Discussion. AMG probably exerts its action on glutamic acid utilization in *L. arabinosus* chiefly by inhibiting the synthesis of glutamine, while GGH acts by inhibiting the utilization of glutamine in subsequent reactions(1-3). The inhibitory action of the comparable aspartic acid analogues, AMA and AGH, is probably different since asparagine is not utilized as well as aspartic acid and appears to play no significant role in growth on aspartic acid under these conditions(4). It is planned to test these substances in bacterial systems in which asparagine is necessary for rapid growth in media containing aspartic acid. Further studies are being made of the mechanism of inhibition of growth by the above compounds.

Substances other than those above which have been shown previously to inhibit utilization of aspartic acid by microorganisms are cysteic acid(5), DL-*para*-hydroxyaspartic acid(6), *meso*-diaminosuccinic acid(6), α-aminolevulinic acid(7), and heated aspartophenone(7).

Summary. β-DL-Aspartylhydrazide and α-DL-methylaspartic acid were found to be good inhibitors of the utilization of L-aspartic acid for the growth of *L. mesenteroides* P-60. The specificity of the inhibition was shown by the finding that the corresponding analogues of glutamic acid were much less active.

---

1. Roper, J. A., and McIlwain, H., *Biochem. J.*, 1948, v42, 485.

2. Ayengar, P., and Roberts, E., PROC. SOC. EXP. BIOL. AND MED., 1953, submitted for publication.

3. ———, PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 476.

4. Hac, L. R., and Snell, E. E., *J. Biol. Chem.*, 1945, v159, 291.

5. Ravel, J. M., and Shive, W., *J. Biol. Chem*, 1946, v166, 407.

6. Shive, W., and Macow, J., *J. Biol. Chem.*, 1946, v162, 451.

7. Dittmer, K., *Ann. N. Y. Acad. Sci.*, 1950, v52, 1274.

Received July 3, 1953. P.S.E.B.M., 1953, v83.

Circulating 17-Hydroxycorticosteroids in Ascorbic Acid-Deficient Guinea Pigs.* (20473)

ALAN K. DONE, ROBERT S. ELY, LAWRENCE R. HEISELT, AND VINCENT C. KELLEY.
From the Lockhart Memorial Laboratory, Department of Pediatrics, University of Utah College of Medicine, Salt Lake City, Utah.

Since the demonstration by Szent-Györgyi (1) of high concentrations of ascorbic acid in the adrenal gland, numerous controversial reports concerning the relation of ascorbic acid to the pituitary-adrenal system and its secretions have appeared. These were recently reviewed by Pirani(2). The development of a technic for the quantitation of circulating 17-hydroxycorticosteroids(3) has permitted a more direct approach to this problem than had been previously available. The data presented in this report were obtained in preliminary studies of circulating 17-hydroxycorticosteroids in guinea pigs rendered ascorbic acid-deficient.

Methods. Male guinea pigs weighing 500 to 750 g were used in these experiments. Ascorbic acid deficiency was induced by a scorbutigenic diet described by Oesterling and Long(4). This diet was given *ad libitum* and the animals were weighed at regular intervals. In most instances a stabilization of weight curves occurred within a few days; rapid weight loss occurred after 14 to 18 days. Only those animals who exhibited a stabilization of weight for at least 10 days prior to the late weight loss and who gave no clinical indications of infection are included in this report. A group of animals who had exhibited a profound weight loss of 2 to 3 days' duration prior to the 17th day of scorbutigenic diet were treated with crystalline ascorbic acid, given orally in a dose of 25 mg per animal per day, from the 17th day until sacrifice on the 22nd day. Each of these animals responded with a moderate weight gain within one to 2 days. *Ad libitum* controls were maintained on a Purina pellet ration. All animals had free access to tap water. At the time of sacrifice, animals were rapidly anesthetized in an ethyl ether atmosphere and blood samples were obtained from the abdominal aorta into heparinized syringes. Immediately after exsanguination of the animal, adrenals were removed, cleaned, weighed and homogenized in 4% trichloroacetic acid. Plasma samples were separated by centrifugation and stored in deep-freeze until used. In some instances the plasmas of 2 to 3 guinea pigs were pooled to make 10 to 15 ml samples for 17-hydroxycorticosteroid determinations.

Circulating 17-hydroxycorticosteroid concentrations were determined by the plasma method of Nelson and Samuels(3). Adrenal ascorbic acid content was measured by the method of Roe and Kuether(5).

Observations. The duration of ascorbic acid-deficient intake and the severity of resultant weight loss are listed for each animal in Table I. As will be seen in this table, prolonged ascorbic acid deficiency resulted in the nearly complete depletion of adrenal ascorbic acid and a striking elevation of circulating 17-hydroxycorticosteroid concentrations. In the group of animals who developed severe ascorbic acid deficiency as evidenced by weight curves, but were treated with ascorbic acid for 5 days prior to sacrifice, the adrenal ascorbic acid content as well as plasma concentrations of 17-hydroxycorticosteroids were not significantly different from *ad libitum* controls (Table II).

Discussion. Exceedingly high plasma concentrations of 17-hydroxycorticosteroids were found in animals whose adrenal glands were almost completely devoid of ascorbic acid. This would suggest that if ascorbic acid is in any way essential to the formation of 17-hydroxycorticosteroids, it need be present only in minute amounts in the adrenal cortex. Concentrations of steroids reported here do not necessarily reflect an accelerated rate of adrenal cortical secretion, but may be the

---

* This research was supported, in part, by a grant from the Helen Hay Whitney Foundation.

TABLE I. Plasma 17-Hydroxycorticosteroids in Guinea Pigs with Treated and Untreated Ascorbic Acid Deficiency.

	No. days on diet	Severity of scurvy*	Adrenal wt (mg/100 g body wt)	Adrenal ascorbic acid (mg/100 g adrenal)	Plasma 17-hydroxycorticosteroids (μg/100 ml)
Scorbutigenic diet	24	+	82	1.11	152.5
	24	+	114	.84	
	24	++	103	.90	
	22	++	108	1.23	380.0
	22	++	71	1.32	
	21	++	127	.84	478.0
	21	++	88	.99	153.0
	21	++	100	.72	124.0
	16	+++	115	.60	222.2
	22	+++	66	1.20	394.2
	22	+++	151	.99	
	22	+++	99		
	21	+++	101	.84	—
	22	+++	147	.51	526.0
	21	+++	98	1.08	—
	25	+++	101	.81	192.8
	24	+++	115	.57	—
	21	+++	105	.63	—
	23	++++	122	.84	282.1
	25	++++	142	.51	416.0
Mean	22		108 ± 5.1	.87 ± .018	301.9 ± 43.03
Scorbutigenic diet (ascorbic acid, 25 mg/day for last 5 days)	22	++	47	37.8	43.3
	22	++	84	39.0	65.4
	22	++	83	26.6	62.5
	22	++	65	42.0	30.5
	22	++++	119	33.7	141.8
Mean	22		80 ± 12.0	35.8 ± 2.66	68.7 ± 19.35
Ad libitum controls	Mean		47 ± 3.0	35.5 ± 3.86	32.9 ± 4.60
	No. animals		10	10	17

* Severity of scurvy was classified on the basis of weight response curves (extent and rapidity of late weight loss) without regard to laboratory data. In the ascorbic acid treated group the classification was based on the status of the animals before treatment was started.

result of interference with the utilization, conjugation or excretion of 17-hydroxycorticosteroids. Ascorbic acid deficiency of the severity produced in these experiments undoubtedly constitutes a chronic stressful situation which may account, wholly or in part, for the excessive amounts of circulating 17-hydroxycorticosteroids observed. However, irrespective of the initiating circumstances, it would appear that these steroids can be produced in the virtual absence of ascorbic acid and that adrenal cortical insufficiency, at least from the standpoint of the ability to produce 17-hydroxycorticosteroids, does not result from a depletion of adrenal ascorbic acid. Whatever the nature of the abnormality responsible for the excessively high plasma concentrations of 17-hydroxycorticosteroids noted, it is largely corrected by the administration of excessive amounts of ascorbic acid over a brief period.

TABLE II. Probability Values, Plasma 17-hydroxycorticosteroids in Guinea Pigs with Treated and Untreated Ascorbic Acid Deficiency.

Scorbutigenic diet	vs. *ad libitum* controls	p <.01
	vs. scorbutigenic diet + ascorbic acid	p <.01
Scorbutigenic diet + ascorbic acid	vs. *ad libitum* controls	p .09

In an attempt to evaluate the role of various factors involved, further experiments are in progress utilizing pair-fed controls, adrenalectomized controls, varying schedules of ascorbic acid administration, and less severe ascorbic acid deficiency; these include urinary excretion of these steroids.

Summary. 1. Quantitative determinations of plasma 17-hydroxycorticosteroids in guinea pigs with severe ascorbic acid deficiency were made. 2. Plasma concentrations of these steroids were found to be approximately 10 times those found in normal guinea pigs despite the virtual absence of adrenal ascorbic acid. 3. Scorbutic animals treated with ascorbic acid for 5 days prior to sacrifice had circulating 17-hydroxycorticosteroids in amounts not significantly higher than those of control animals.

The authors are indebted to Robert O. Hickman, Doris F. Tippit and H. Beth Dowding for technical assistance.

---

1. Szent-Györgyi, A., *Biochem. J.*, 1928, v22, 1387.
2. Pirani, C. L., *Metabolism,* 1952, vI, 197.
3. Nelson, D. H., and Samuels, L. T., *J. Clin. Endocrinol. and Metab.*, 1952, v12, 519.
4. Oesterling, M. J., and Long, C. N. H., *Science,* 1951, v113, 241.
5. Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 1943, v147, 399.

Received July 6, 1953. P.S.E.B.M., 1953, v83.

Effects of Chloramphenicol on Incorporation of Glycine-2-C^{14} into Mammalian Tumor Cell Proteins and Purines.* (20474)

G. A. LePage (Introduced by H. P. Rusch)

From the University of Wisconsin Medical School, McArdle Memorial Laboratory, Madison.

It has been demonstrated by Wisseman *et al.*(1) that the antibiotic d(—) *threo* chloramphenicol is effective in inhibiting protein synthesis in susceptible microorganisms such as *E. coli.* They have further demonstrated that higher concentrations of 1(+) *threo* chloramphenicol do not give this effect, and that concentrations of the d(—) threo isomer which effectively stop protein synthesis (50 μg/ml) permit nucleic acid purine and ribose synthesis to proceed for a significant time. Since viable suspensions of mammalian tumor cells were available and had been shown to carry on incorporation of glycine-2-C^{14} into purines and proteins(2), it was of some interest to test the effects of chloramphenicol isomers on this mammalian cell system.

Methods. The Ehrlich carcinoma cells were obtained by intraperitoneal injection of 6-10-week-old Rockland strain white mice with approximately 10^7 cells per mouse. The cells were withdrawn from mice after 5-7 days, under light ether anesthesia with a hypodermic syringe and No. 18 needle. At this time the suspension was approximately 90% tumor cells. The Gardner lymphosarcoma (6C3HED) cells were obtained in similar fashion from C3H mice 7-10 days after injection, at which time the suspensions were approximately 96% tumor cells. The cells were washed 3-4 times by suspending in isotonic saline and centrifuging. Warburg respirometer vessels of 60 ml capacity were used as reaction vessels. Incubation was for one hour at 38°C. Each vessel contained a total of 12 ml of medium, consisting of Robinson's medium with glucose and bicarbonate(3), 150 μg glycine-2-C^{14}† and cells equivalent to 30 mg dry weight. The chloramphenicols‡ were weighed dry into each such flask and dissolved in the medium. The cells were added just prior to incubation, except where otherwise indicated. Purines and proteins were isolated and counted for radioactivity as described earlier(2). Paper spots as counted contained 200-300 μg of purine. Counting was continued until statistically significant to within 5%.

---

* This research was supported in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council and in part by the Alexander and Margaret Stewart Fund.

† Specific activity 20,600,000 counts/min/mg; obtained from Tracerlab, Inc., on allocation by the U. S. Atomic Energy Commission.

‡ The d(-) threo chloramphenicol and 1(+) threo chloramphenicol were obtained through the courtesy of Parke-Davis and Co. and Major Charles L. Wisseman, Jr., respectively.

TABLE I. Incorporation of Glycine-2-C^{14} into Components of Ehrlich Carcinoma Cells in the Presence of Various Concentrations of d(—) Threo Chloramphenicol.

Chloramphenicol, μg/ml	Protein		Nucleic acid adenine		Nucleic acid guanine	
	Radioactivity, c/min./mg	% inhibition	Radioactivity, c/min./mg	% inhibition	Radioactivity, c/min./mg	% inhibition
0	3970		1280		2770	
250	3550	10	1340	–5	2970	–7
500	3570	10	1390	–9	2720	2
1000	2950	26	1120	12	1760	36
1500	2700	32	790	38	1110	60
2500	2645	33	770	40	1100	60

Radioactivities are corrected for self-absorption characteristics. Each figure is the mean of results from 3 experiments with duplicate reaction vessels.

TABLE II. Effects of Chloramphenicol Isomers on Incorporation of Glycine-2-C^{14} by Ascites Cell Tumors *In vitro.*

Addition, μg/ml	Radioactivity, c/min./mg		
	Protein	Adenine	Guanine
	Ehrlich carcinoma		
0	3990	1290	2980
1000 d(—) threo	3190	1060	1875
2000 d(—) "	2710	1005	1280
1000 l(+) "	3670	1295	1910
2000 l(+) "	3790	1120	1105
1000 d(—) " *	3150	1030	1875
1000 l(+) " *	3750	1070	1250
	6C3HED		
0	3650	1050	1210
1000 d(—) threo	1270	965	361
2000 d(—) "	310	757	182
1000 l(+) "	1460	988	556
2000 l(+) "	184	965	194

* Preincubated 30 min. at 0°C.

Each figure represents avg of results in 3 to 5 experiments in the Ehrlich carcinoma, 2 experiments in the 6 C3HED.

Results and discussion. Data are presented in Table I to show the effects of varied levels of d(—) *threo* chloramphenicol upon the incorporations by Ehrlich carcinoma cells. Significant effects are obtained only at much higher levels than are required to inhibit susceptible bacteria. In Table II are data in which the 2 chloramphenicol isomers are compared in experiments with 2 types of tumor cells. In experiments with Ehrlich carcinoma cells the inhibition of incorporation of the radioactivity into protein is significant only with the isomer that has antibiotic properties. This specificity does not hold for the lymphosarcoma cells. The level of inhibition varied considerably from one experiment to another in the Ehrlich carcinoma cells, but the relative extent of inhibition of the 3 components studied held constant. This raised the question as to whether the permeability of the cells to the drug was a variable. As seen in Table II, preincubation of the cells with the drugs at 0°C did not increase the inhibition.

The results obtained by Tyner *et al.*(4) (Chart 16), indicated that synthesis of the individual nucleotide components of tumor nucleic acids was at a rate in each case parallel with the requirement for that nucleotide in the nucleic acid composition. The inhibition of guanine synthesis, without a comparable inhibition of other synthetic processes involved in growth, might lead to interference with the growth process as a whole, possibly by leading to modified nucleic acid composition. In this connection it is of interest that triethylene melamine, which is known to be an inhibitor of a number of animal tumors, inhibited the incorporation of glycine-2-C^{14} into nucleic acid guanine of Ehrlich carcinoma cells 66% at 30 μg per ml. The incorporation into protein and into nucleic acid adenine was not affected. It would be appropriate to test the *in vivo* effect of these isomers of chloramphenicol on various tumors.

It is worthy of note that when the incorporation of glycine into bacterial purines was finally inhibited, the entry into both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) purines was affected(1). The 2 nucleic acids were not separated in the experiments reported here and a 60% inhibition of incorporation of glycine-2-C^{14} into guanine may well represent complete blocking of entry into DNA.

Summary. The effects of 2 of the isomers of chloramphenicol, one the antibiotic, the other inactive toward bacteria, were tested on 2 *in vitro* systems consisting of mouse ascites

tumor cells. In Ehrlich carcinoma cells the antibiotic isomer inhibited the incorporation of glycine-2-C^{14} into protein and nucleic acid purines. The other isomer inhibited incorporation into the purines to the same extent, but did not appreciably affect entry into proteins. In lymphosarcoma cells (6C3HED), both isomers were equally effective in inhibiting the incorporation into proteins and purines. Incorporation into guanine was inhibited much more than into adenine. It is suggested that this might indicate tumor inhibiting properties. The levels of the antibiotic necessary to give significant inhibition are much higher than are required to affect bacteria.

1. Wisseman, C. L., Jr., Hahn, F. E., Hopps, H., and Smadel, J. E., *Fed. Proc.*, 1953, v12, 466.

2. LePage, G. A., *Cancer Res.*, 1953, v13, 178.

3. Robinson, J. R., *Biochem. J.*, 1949, v45, 68.

4. Tyner, E. P., Heidelberger, C., and LePage, G. A., *Cancer Res.*, 1953, v13, 186.

Received July 6, 1953. P.S.E.B.M., 1953, v83.

Some Interrelations of Dietary Protein, Molybdenum, Riboflavin and Calories on Liver and Intestinal Xanthine Oxidase.* (20475)

DAN A. RICHERT AND W. W. WESTERFELD

From the Department of Biochemistry, State University of New York, Medical College at Syracuse.

Previous studies(1-3) have described the dependence of liver and intestinal xanthine oxidase upon the dietary intake of protein, the xanthine oxidase factor, riboflavin(4) and calories(2). The additional studies herein reported on the interrelationship of the various factors were made possible by the identification of the xanthine oxidase factor as the trace element molybdenum(5,6). The following questions were studied. 1) How much does a "pure" deficiency of riboflavin and/or molybdenum affect the concentration of xanthine oxidase in the liver and intestine, and does a specific deficiency of either factor alter the oxidase:dehydrogenase ratio of the enzyme? 2) Is the loss of xanthine oxidase from these tissues during starvation due to an inadequate protein intake, the caloric restriction, or a combination of such factors? 3) Does the molybdenum content of the diet influence the protein effect on tissue xanthine oxidase? The latter question has a bearing on the use of liver xanthine oxidase concentrations as a criterion of the dietary value of a protein(7).

Experimental. Riboflavin, Mo and calories. Weanling male albino rats were divided into 4 groups and fed purified diets which were identical in composition with the one previously described(1), except that the diets contained 24% Labco casein and 65% glucose. The diets were varied by adding or omitting molybdenum and riboflavin, as shown with the results. When present, riboflavin and Mo (as Na_2MoO_4) were added to the diet at 4 and 1 mg per kg, respectively. These diets were fed *ad libitum,* and the daily food consumption of the rats consuming the control diet No. 4, containing riboflavin and Mo, was measured. A fifth group of rats was fed the same diet but was given daily only 23% of the total food intake eaten by the control group. A sixth group of rats was fed a diet containing 96% casein, 4% salts, the vitamin supplement and 1 mg Mo per kg diet, but these rats received daily only 25% of the amount of food eaten by the control group. These intakes were established so that Group 6 would receive the same daily caloric intake as Group 5 and the same daily protein intake as the control Group 4. After feeding the diets for 2 to 3 weeks the livers and intestines were analyzed for xanthine oxidase in the presence and absence of methylene blue by the methods previously described(2). The results are shown in Table I.

---

* This study was aided by a grant from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

TABLE I. Effect of Variations in Dietary Riboflavin and Molybdenum on Liver and Intestinal Xanthine Oxidase in Weanling Rats.

	Diet		Days	Body wt		No. of	Liver XO†		Intestinal XO†	
No.	B_2	Mo	on diet	Start	End	rats	—	+MB	—	+MB
1	+	—	17	50	134	8	24 ± 2.4	38 ± 4.1	3 ± 1.0	12 ± 2.7
2	—	+	14	51	67	7	26 ± 3.0	42 ± 3.8	21 ± 2.7	40 ± 3.1
3	—	—	14	47	57	6	28 ± 2.6	42 ± 2.9	3 ± 1.7	15 ± 2.8
4	+	+	17	51	104	8	27 ± 2.9	47 ± 3.8	39 ± 3.2	58 ± 4.6
6*	+	+	13	52	39	3	25	39	39	64

* A 96% casein diet fed at 25% of level of diet 4 eaten *ad libitum.* Diets 1-4 contained 24% casein.

† Liver and intestinal xanthine oxidase values, determined in presence and absence of methylene blue (MB), have been recorded as net cmm O_2 consumed per 20 min. per Warburg flask containing 283 mg fresh tissue; values are the mean ± stand. error of the mean.

TABLE II. Lack of Effect of Partial Caloric Restriction, with and without a Simultaneous Reduction in Protein Intake, on Rat Liver and Intestinal Xanthine Oxidase.

	Diet									
No.	Casein content, %	Amt fed, %*	Days fed	Body wt, g Start	End	No. of rats	Liver XO† —	+MB	Intestine XO† —	+MB
4	24	100	28	120	239	13	26±1.8	47±2.5	32±1.8	48±2.5
5	24	23	22	119	85	13	31±4.2	46±5.2	35±3.5	56±3.8
6	96	25	20	125	90	10	26±2.2	44±3.7	37±2.4	57±3.9

* *Ad libitum* consumption of 24% casein diet was taken as 100% (Group 4), and Groups 5 and 6 were fed 23% and 25% respectively of this amount daily.

† Liver and intestinal xanthine oxidase values, determined in presence and absence of methylene blue (MB), have been recorded as net cmm O_2 consumed per 20 min. per Warburg flask containing 283 mg fresh tissue; values are the mean ± stand. error of the mean.

Xanthine oxidase was removed extensively from the intestine on molybdenum deficient diets whether riboflavin was present or not. When the diet contained both of these constituents, saturation levels of the intestinal enzyme were obtained, but only half as much enzyme was present when flavin was omitted. Calculation(8,9) of the oxidase:dehydrogenase ratio of the liver or intestinal enzyme showed that by this criterion the composition of the enzyme was unchanged by dietary variations in riboflavin and molybdenum. In this experiment a deficiency of riboflavin and/or molybdenum had no effect on the concentration or oxidase:dehydrogenase ratio of liver xanthine oxidase. Additional rats that had been on these diets for 4 to 5 weeks had intestinal xanthine oxidase values which were essentially identical with those shown in Table I.

Since all of the rats in Group 5 and most of the rats in Group 6 died as a result of the caloric restriction within the first 2 weeks, this portion of the experiment was repeated with older rats. The daily food consumption of Group 4, which was fed the purified 24% casein diet containing riboflavin and Mo, was measured, and 23% of this amount was fed to Group 5; 25% of this amount of the 96% casein diet was fed to Group 6. The results are shown in Table II.

Both of the groups receiving the restricted food intake did not lose xanthine oxidase from either the liver or the intestine. Complete starvation caused a loss of this enzyme from both tissues(2) but a reduction in calories to ¼ the usual food intake had no effect. This restriction of the 24% casein diet reduced the absolute daily protein intake to less than that provided by the *ad libitum* feeding of an 8% casein diet; the latter caused a marked and almost complete loss of this enzyme from both the liver and intestine(1), but when the calories were restricted simultaneously, the tissue concentration of xanthine oxidase was unaffected. An obvious difference exists between the effect of protein restriction in the presence of adequate calories and the simultaneous restriction of both protein and calories.

TABLE III. Effect of Variations in Dietary Molybdenum and Protein on Liver and Intestinal Xanthine Oxidase.

Dietary casein, %		35		24		18		8		0	
Dietary Mo, γ/kg		40	1000	40	1000	40	1000	40	1000	40	1000
Avg days on diet		21	23	23	23	19	22	23	23	13	13
Final body wt, g*		107	110	107	104	97	109	53	56	32	31
No. rats analyzed		11	14	14	12	9	11	12	12	7	7
Xanthine oxidase†	Liver	27±2.6	28±1.8	21±2.0	19±2.0	15±2.7	15±3.6	3±1.2	4±1.8	9±2.7	5±3.9
	Intestine	24±2.2	40±2.2	25±2.1	30±2.5	18±4.9	27±4.5	12±1.9	14±2.1	13±1.5	19±3.4

* Starting body wt averaged 37-39 g for all groups.

† Liver and intestine xanthine oxidase activities have been recorded as net cmm O_2 consumed per 20 min. per Warburg flask containing 283 mg fresh tissue; values are mean ± stand. error of the mean.

Protein and Mo. Weanling male rats were fed diets varying in protein content from 0 to 35% casein; each such diet contained 4 mg riboflavin and either 40 γ or 1000 γ Mo (as Na_2MoO_4) per kg. The diets were otherwise identical with the one previously described; variations in the casein content were made with corresponding adjustments of the glucose concentration. After being on the diets for 2 to 4 weeks, the rats were sacrificed and analyzed for liver and intestinal xanthine oxidase. The results are shown in Table III.

The dominant role of dietary protein in controlling the level of liver xanthine oxidase was again evident. Diets containing either 1 mg or 0.04 mg Mo per kg gave the same level of liver enzyme at each protein concentration. Hence large increases in the molybdenum content of the diet did not alter the response of liver xanthine oxidase to dietary protein. Low protein diets prevented the intestinal xanthine oxidase from responding normally to either 40 or 1000 γ of Mo/kg, but much higher levels of intestinal enzyme were obtained on a protein-free-Mo-containing diet than on an adequate protein intake without molybdenum. Hence the molybdenum content of the diet tended to dominate the control of the level of intestinal xanthine oxidase, just as protein dominated the liver enzyme. Since a dietary concentration of 40 γ Mo/kg was not enough to produce saturation levels of intestinal xanthine oxidase (*i.e.*, above 30), the diets containing adequate protein and 1000 γ Mo/kg all gave higher levels of this enzyme in the intestine. Large amounts of dietary molybdenum did not give saturation levels of intestinal xanthine oxidase in the absence of an adequate protein intake.

Summary. The omission of riboflavin and/or molybdenum from a 24% casein diet fed to weanling rats had no effect on liver xanthine oxidase within 2 weeks; a molybdenum deficiency in the presence or absence of riboflavin removed xanthine oxidase from the intestine almost completely, while a riboflavin deficiency in the presence of molybdenum gave levels of intestinal enzyme which were about one-half the saturation levels achieved by a diet containing both molybdenum and riboflavin. The oxidase:dehydrogenase activity of the enzyme present in the liver or intestine was not altered by a riboflavin or molybdenum deficiency. Restriction of the daily food intake to 23% of the *ad libitum* intake of a 24% casein diet or 25% of a 96% casein diet had no effect on liver or intestinal xanthine oxidase. The level of liver xanthine oxidase depended primarily on the protein content of the diet, and such values were the same whether the diet contained 0.04 or 1 mg of molybdenum per kg. Intestinal xanthine oxidase levels were determined primarily by the molybdenum content of the diet, but low protein diets allowed only intermediate levels of this enzyme to be achieved even in the presence of a large excess of molybdenum.

---

1. Westerfeld, W. W., and Richert, D. A., *J. Biol. Chem.*, 1950, v184, 163.

2. ———, *J. Biol. Chem.*, 1951, v192, 35.

3. Richert, D. A., and Westerfeld, W. W., *J. Biol. Chem.*, 1951, v192, 49.

4. Doisy, R. J., and Westerfeld, W. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 203.

5. De Renzo, E. C., Kaleita, E., Heytler, P., Oleson, J. J., Hutchings, B. L., and Williams, J. H., *J. Am. Chem. Soc.*, 1953, v75, 753.

6. Richert, D. A., and Westerfeld, W. W., *J. Biol. Chem.*, 1953, v203, 915.

7. Williams, J. N., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, v181, 559.

8. Meikleham, V., Wells, I. C., Richert, D. A., and Westerfeld, W. W., *J. Biol. Chem.*, 1951, v192, 651.

9. Westerfeld, W. W., and Richert, D. A., *J. Biol. Chem.*, 1952, v199, 393.

Received July 8, 1953. P.S.E.B.M., 1953, v83.

Biological Half-Life of Endogenous ACTH.* (20476)

KATHERINE L. SYDNOR AND GEORGE SAYERS.

From the Department of Pharmacology, College of Medicine, University of Utah, Salt Lake City, and the Department of Physiology, Western Reserve University School of Medicine, Cleveland, O.

The relatively high concentration of adrenocorticotrophic hormone (ACTH) in the blood of the adrenalectomized rat(1,2) makes it possible to determine the biological half-life of endogenous hormone in this animal.

Methods. Male rats from the Holtzman-Rolfsmeyer farm weighing 200 to 300 g were acclimatized for 2 weeks in the laboratory (constant lighting and constant temperature, 76 ± 2°F) before use in the experiments.

Two separate experiments were performed to determine the rate of disappearance of ACTH from the blood of adrenalectomized rats following hypophysectomy. One week (Exp. 1) or 2 weeks (Exp. 2) after bilateral adrenalectomy,[†] the animals were anesthetized with ether and either sham-hypophysectomized (pituitary exposed, but not removed) or hypophysectomized. The sham-hypophysectomized animals served as zero-time controls. All rats were decapitated[‡] and blood was drained from the trunk into 4 volumes of glacial acetic acid. Blood samples were collected exactly one minute after sham-hypophysectomy, and 1, 2, or 3 minutes after hypophysectomy. The blood-glacial acetic acid mixtures from individual rats of each group were pooled and processed by the oxycellulose[§] method(3).

Exp. 3 was designed to determine if the rapid disappearance of ACTH from the blood of adrenalectomized rats one minute after hypophysectomy could be attributed to inactivation in blood itself. Two weeks after bilateral adrenalectomy, 76 rats were exposed to ether for 2 minutes, then bled from the abdominal aorta for a period of one minute. One-third of each blood sample was added immediately to 4 volumes of glacial acetic acid; the remaining two-thirds were quickly transferred to a centrifuge tube, incubated for one minute at 37°C, and then immediately added to 4 volumes of glacial acetic acid. The non-incubated (control) and the incubated blood-glacial acetic acid mixtures were pooled separately and processed by the oxycellulose method.

The pH of the HCl-eluate of each pool of processed blood was adjusted to 4 immediately before assay for ACTH in male hypophysectomized rats by the adrenal ascorbic acid-depletion method(4). The estimate of the concentration of ACTH in blood is based on the assumption that the oxycellulose method recovers 100% of the hormone. Studies on ACTH added to hypophysectomized rat blood indicate that 96 ± 6% of the hormone is recovered(5).

---

* This investigation was supported in part by a research grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, and in part by a research grant from the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, Contract No. DA-49-007-MD-180.

[†] Adrenalectomized animals were given 0.9% sodium chloride solution to drink.

[‡] Decapitated in order to prevent the adenohypophysis of the sham-hypophysectomized rat from contributing to blood ACTH during bleeding.

[§] Samples of oxidized cellulose (10-12% COOH) were generously supplied by the Research Laboratories, Tennessee Eastman Co.

TABLE I. Blood ACTH.

Exp. No.	Test substance	Dose (ml per 100 g test rat)	Ascorbic acid depletion (mg/100 g adrenal): Individual responses*	Avg	Estimate of conc., M ± S.E.M., mu† per 100 ml
1	U.S.P. ACTH Std.	1.0 mu	112, 80, 103, 145, 117	111	
		.25	43, 57, 34, 27, 50	42	
	Adrenx rat blood, 1′ after sham-hypox	6.0 ml	50, 122, 82, 109	104	11.8 ± 2.5
		3.0	84, 30, 61	58	
		1.5	27, 24, 27, 52	33	
	Adrenx rat blood, 1′ after hypox	12.0 ml	111, 93, 109, 87, 88	98	6.0 ± 1.3
		6.0	46, 55, 40, 63	54	
		3.0	15, 13, 82	37	
	Adrenx rat blood, 2′ after hypox	12.0 ml	74, 76, 35	86	3.8 ± .9
		6.0	31, 45, 20, 36, 30	32	
		3.0	5, 3, –28, 5, 10	–1	
	Adrenx rat blood, 3′ after hypox	12.0 ml	65, 48, 118, –16	77	2.8 ± 1.2
		6.0	48, 23, 35, 40	36	
		3.0	42, –25, 16, –36, 36	7	
2	U.S.P. ACTH Std.	1.0 mu	188, 157, 160, 137, 145, 170	160	
		.5	102, 80, 125, 107, 69, 69	92	
		.25	63, 37, –4, 87, 77, 86	58	
	Adrenx rat blood, 1′ after sham-hypox	6.0 ml	124, 112, 134, 142, 107	124	11.0 ± 1.7
		3.0	54, 97, 50, 71, 95, 82	75	
		1.5	2, 98, 44, 77, 18, 42	47	
	Adrenx rat blood, 1′ after hypox	12.0 ml	90, 105, 118, 145, 58, 75	98	3.8 ± .6
		6.0	45, 76, 61, 56	60	
		3.0	27, 17, –34, 36, –2	9	
	Adrenx rat blood, 3′ after hypox	24.0 ml	55, 108, 121, 123, 119	105	2.0 ± .6
		12.0	34, 65, 38, 68, 18, 11	40	
		6.0	9, 65, 31, –7	25	
3	U.S.P. ACTH Std.	1.0 mu	140, 122, 171, 102, 132, 122, 136	131	
		.5	95, 64, 89, 80, 129, 127, 128	102	
		.25	49, 56, 65, 111, 105, 71	76	
	Adrenx rat blood, (control)	6.0 ml	105, 103, 222, 165, 128	145	13.7 ± 3.0
		3.0	44, 121, 138, 40, 55, 52, 80	80	
		1.5	83, 106, 50, 55, 32, 61, 20	58	
	Adrenx rat blood (incubated 1′ at 37°C)	12.0 ml	150, 124, 196, 178, 169	163	13.6 ± 2.7
		6.0	153, 116, 135, 138, 146, 85	129	
		3.0	33, 93, 81, 128, 85	84	

* Difference in ascorbic acid concentration in right adrenal removed prior to injection of test substance and concentration in left adrenal removed 1 hr after injection.

† One mu equals one-thousandth of a U.S.P. unit of ACTH.

Results. The individual and average responses (adrenal ascorbic acid depletion) to the test material and to the Standard ACTH assayed on the same day, as well as the estimates of blood-ACTH concentrations, are presented in Table I. The data indicate that approximately 75% of the biological activity disappears from the blood within 3 minutes after hypophysectomy. Estimates of biological half-life were obtained by plotting the logarithm of the concentration of blood-ACTH against time, and by calculating the lines of best fit by the method of least squares (Fig. 1). The estimates for the 2 experiments, 0.95 and 1.25 minutes, are in good agreement.

The results of Exp. 3 indicate there is no appreciable loss of biological activity when adrenalectomized rat blood is incubated *in vitro* for one minute at 37°C. The estimate of the blood-ACTH concentration of the control (non-incubated) blood is not significantly different from that of the incubated blood. It appears that the rapid disappearance of

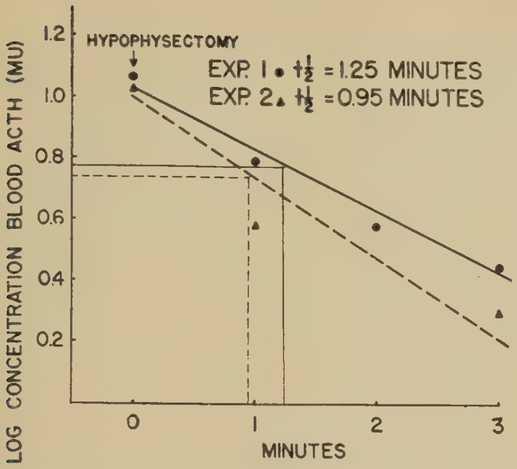


FIG. 1. Rate of disappearance of ACTH from blood of adrenalectomized rats following hypophysectomy (zero-time). See text for explanation.

ACTH from the blood of adrenalectomized rats one minute after hypophysectomy cannot be attributed to destruction of ACTH in blood itself.

Discussion. The 2 estimates of biological half-life of endogenously produced ACTH, 0.95 and 1.25 minutes, are in good agreement. The difference in the 2 estimates may be explained on the basis of the error of the bioassay method for the analysis of ACTH rather than on incomplete or inconsistent recovery of the hormone from blood by the oxycellulose method. Actually, incompleteness of recovery would have little, whereas inconsistency of recovery would have a significant, influence upon the estimation of the half-life of ACTH. The results of 7 separate experiments on recovery of Standard ACTH added to hypophysectomized rat blood indicate that the oxycellulose method recovers 96 ± 6% of the hormone(5). An analysis of the results of 14 separate experiments indicates that the mean concentration of ACTH in blood of acutely exsanguinated, adrenalectomized rats with intact pituitaries is 12.8 ± 0.6 mu per 100 ml. The small standard errors of these estimates provide strong evidence for consistency of recovery of endogenous ACTH by the oxycellulose method.

No previous reports have appeared in the literature regarding the biological half-life of endogenous ACTH. Van Dyke *et al.*(6) have calculated the rate of disappearance of endogenous ACTH and have estimated the ". . . average life of the ACTH hormone molecule in the circulation of the rat at physiological levels" to be approximately 17 minutes. This estimate was based on adrenal weight changes in parabiotic rats, a bioassay technic which is quantitatively inadequate to measure ACTH. The estimate of the half-life of ACTH in the present study has been made, necessarily, in adrenalectomized rats. It is not technically feasible to determine the half-life in rats with intact adrenal glands. The concentration of ACTH in blood of the intact rat is too low, even under conditions of stress, to make a quantitative analysis of the type presented in this report. If the adrenals "utilize" ACTH, and on this point there are no definitive data, one would expect the half-life to be even shorter in the intact than in the adrenalectomized animal.

Endogenously produced ACTH appears to have a shorter half-life than exogenous ACTH. Values of 5.5 minutes(7) and 5 minutes(1) have been reported for sheep ACTH administered to intact rats. Sheep ACTH apparently has the same half-life in adrenalectomized rats(1). Calculation of the data presented by Richards and Sayers(8) indicate that the half-life of rat ACTH administered to intact rats is approximately 2 minutes, a value in close agreement with the estimates for endogenous ACTH presented in this study. The reason for the difference between the half-life of injected sheep and injected rat ACTH is not apparent. It is reasonable to assume that a study of endogenously secreted ACTH has more physiological significance.

The mechanisms concerned with the rapid disappearance of ACTH from the circulation are unknown. Inactivation in blood itself cannot be a major factor. Experiments on renal excretion of administered ACTH in rat(8) and man(9) indicate that no appreciable fraction of the hormone is excreted in biologically active form. However, no quantitative data are available on renal excretion of endogenous ACTH. ACTH is rapidly inactivated when incubated with slices or homogenates of various tissues(10). *In vitro* studies of this nature are difficult to interpret in physiological terms, since disintegrated cells exhibit an abnormally high degree of proteolytic activity. For example, intact pituitaries incubated for one hour at 37°C show no loss of ACTH activity, whereas crushed pituitaries lose practically all activity within a few minutes after cell injury(11).

Characterization of the dynamics of the pituitary-adrenocortical system would add appreciably to our understanding of the mechanisms which influence the rate of elaboration of ACTH and the cortical steroids. It is now definitely established that ACTH release from the adenohypophysis is accelerated within a remarkably short time after application of a stressful stimulus. The indirect studies of Gray and Munson(12) suggest that acceleration occurs within 10 seconds after application of a stress. Recent direct analyses of blood-ACTH in this laboratory(5) indicate that the blood titer of ACTH is significantly increased within 2 minutes after subjecting animals to stressful stimuli. The present study establishes the fact that ACTH, following its release from the pituitary, has an extremely brief sojourn in the blood.‖ It appears reasonable to conclude that the dynamics of ACTH release and disappearance are characterized by time factors which do not contribute an appreciable degree of inertia to the pituitary-adrenocortical system.

No precise observations have been made on the time-lag between increased rate of release of ACTH and of increased rate of secretion of corticosteroids by the adrenal. The concentration of total 17-hydroxycorticosteroids(13) and of 17-hydroxycorticosterone(14) in blood is significantly increased 15 minutes after a single intravenous injection of ACTH in man. Experiments are in progress in animals to determine with accuracy the time which elapses between accelerated ACTH release and accelerated adrenocorticosteroid secretion.

Another important time-relationship, and the one which may contribute the greatest degree of inertia to the pituitary-adrenocortical system, is the period which elapses between the reduction of the blood-ACTH titer to pre-stress levels and the subsidence of the accelerated secretory activity of the adrenal cortex. Only meagre information is available on this aspect of the problem. Sweat *et al.* (14) found 17-hydroxycorticosterone concentration in blood to remain abnormally high for 4 hours after a single intravenous injection of 15 units of ACTH in man; apparently the secretory activity of the adrenal cortex is accelerated for a relatively long period after a brief stimulus with ACTH. More direct observations have been made by Farrell and Sweat(15) who determined steroid output by the adrenal after hypophysectomy; steroid output at one and 2 hours after hypophysectomy was 50 and 20%, respectively, of preoperative levels.

Finally, the rate of utilization, degradation and excretion of blood adrenocorticosteroids must be determined in order to gain a complete characterization of the dynamics and, in turn, of the regulatory mechanisms of the pituitary-adrenocortical system.

Summary. The biological half-life of endogenous ACTH in blood of the adrenalectomized rat is approximately one minute (0.95 and 1.25 minutes for 2 experiments). No appreciable loss of biological activity occurs when adrenalectomized rat blood is incubated *in vitro* for one minute at 37°C. The rapid disappearance of ACTH from the blood of the adrenalectomized rat one minute after removal of the adenohypophysis cannot be attributed to inactivation of the hormone by blood itself.

‖ It is to be noted that the concentration of ACTH in blood of adrenalectomized animal is greater than that in blood of intact animal. The discussion assumes that rate of disappearance of ACTH from the circulation is the same at all blood concentrations. The validity of the assumption can only be tested when a method is available for determining rate of disappearance of endogenous ACTH at low concentrations.

1. Gemzell, C. A., Van Dyke, D. C., Tobias, C. A., and Evans, H. M., *Endocrinol.*, 1951, v49, 325.

2. Sydnor, K. L., and Sayers, G., *J. Clin. Endocrinol. and Metab.*, 1952, v12, 920.

3. ———, PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 432.

4. Sayers, M. A., Sayers, G., and Woodbury, L., *Endocrinol.*, 1948, v42, 379.

5. Unpublished observations.

6. Van Dyke, D. C., Simpson, M. E., Li, C. H., and Evans, H. M., *Am. J. Physiol.*, 1950, v163, 297.

7. Greenspan, F. S., Li, C. H., and Evans, H. M., *Endocrinol.*, 1950, v46, 261.

8. Richards, J. B., and Sayers, G., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 87.

9. Sayers, G., Burns, T. W., Tyler, F. H., Jager, B. V., Schwartz, T. B., Smith, E. L., Samuels, L. T., and Davenport, H. W., *J. Clin. Endocrinol.*, 1949, v9, 593.

10. Geschwind, I. I., and Li, C. H., *Endocrinol.*, 1952, v50, 226.

11. Burns, T. W., Merkin, M., Sayers, M. A., and Sayers, G., *Endocrinol.*, 1949, v44, 439.

12. Gray, W. D., and Munson, P. L., *Endocrinol.*, 1951, v48, 471.

13. Nelson, D. H., Samuels, L. T., Willardson, D. G., and Tyler, F. H., *J. Clin. Endocrinol.*, 1951, v11, 1021.

14. Sweat, M. L., Abbott, W. E., Jeffries, W. M., and Bliss, E. L., *Fed. Proc.*, 1953, v12, 141.

15. Farrell, G., and Sweat, M. L., unpublished observations.

Received July 9, 1953. P.S.E.B.M., 1953, v83.

Chemical and Biological Studies on *1,2*-Dihydro-*s*-triazines. IV. Activity in Pteroylglutamic Acid-*Streptococcus faecaelis* No. 8043 Systems. (20477)

G. E. FOLEY. (Introduced by Harry Eagle)

From the Laboratories of Microbiology, The Children's Cancer Research Foundation and the Department of Pathology, Harvard Medical School, at Children's Medical Center, Boston, Mass.

Preliminary communications from these laboratories reported the synthesis(1,2) of an hitherto undescribed class of compounds, the *1,2*-dihydro-*s*-triazines which exhibit significant anti-malarial*(1-3), anti-tumor(3), and anti-vitamin(1,4) activity. Details of the synthesis, chemical characterization and identification of these compounds are recorded elsewhere(2,5). During the course of these studies, certain derivatives of this series also have been synthesized independently by others in connection with studies on the identification of the active metabolite of paludrine(6,7).

The biological activity of the symmetrical dihydrotriazines was first observed in these laboratories early in 1950 when the parent-compound of the series (D-19 • HCl) was studied in routine screening assays† for microbiological activity. Since that time, more than 250 samples of 71 derivatives of this series have been studied. The purpose of the present communication is to report the activity of these compounds in *Streptococcus faecalis* No. 8043‡-pteroylglutamic acid (PGA) assay systems.

Methods. PGA assays(8) using *Streptococcus faecalis* were done in Difco PGA Assay Medium or the casein hydrolysate medium prepared as described by Tepley and Elvehjem(9). The basal medium was diluted (usually 1:1) with glass-distilled water to yield uniformly reproducible optical densities of 4.0-6.0 x 10 from minimal inocula with the addition of PGA and no growth in the absence of PGA. Media were handled in chemically clean glassware, dispensed in 10 ml volumes in 22 x 175 mm optically standardized tubes, closed with loose fitting aluminum caps and autoclaved 5 minutes at 15 lb pressure. Crystalline PGA, other metabolites and the various dihydrotriazines were prepared in appropriate concentrations in sterile glass-distilled water and added aseptically to the cooled sterile medium to the desired final concentration. Assays were incubated at 37°C for 24 hours. Optical density was read di-

---

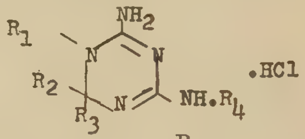
* We are indebted to J. H. Williams and his colleagues of the Lederle Laboratories, Pearl River, N. Y. for the anti-malarial assays.

† The routine screening program, which comprises several standard microbiological assay systems as well as studies with a number of miscellaneous bacteria and fungi was organized in 1947 as one part of a broad program in the chemotherapy of cancer initiated by Sidney Farber.

‡ A. T. C. C. No. 8043.

TABLE I. Inhibition of *Streptococcus faecalis* #8043-PGA Assay Systems by Ketone Derivatives of Dihydrotriazine Series.

Structure (1)



				Inhibition index vs. PGA	
Compound	R_1	R_2	R_3	.01 γ/ml	.001 γ/ml
D-19 (2)	4′-carbethoxyphenyl	methyl	methyl	37.5	325.0
D-20 (2,3,4,5)	4′-chlorophenyl	"	"	9.5	95.0
D-22 (6)	4′-carboxyphenyl	"	"	350000	3500000
D-23 (3,5)	phenyl	"	"	10.0	110.0
D-27 (2)	4′-carboxamidophenyl	"	"	1625	6870
D-32 (1)	phenyl	"	"	33.3	114.0
D-38	2′-tolyl	"	"	4000	12000
D-40	4′-chlorophenyl	pentamethylene		16000	80000
D-43	"	methyl	ethyl	260.0	495.0
D-53	2′,4′-dichlorophenyl	"	methyl	15000	34000
D-54 (3,7)	3′,4′-dichlorophenyl	"	"	10.0	35.0
D-56	2′,5′-dichlorophenyl	"	"	330000	2000000
D-64	4′-chlorophenyl	tetramethylene		460.0	100.0
D-65	4′-bromophenyl	methyl	methyl	12.0	40.0
D-66	phenyl	"	ethyl	240.0	640.0
D-68	2′-chlorophenyl	"	methyl	14100	72400
D-69	3′-chlorophenyl	"	"	32.5	75.0
D-72	2′-tolyl	tetramethylene		310000	800000
D-73	4′-nitrophenyl	methyl	methyl	470.0	4000
D-75	3′,4′-dichlorophenyl	"	ethyl	37.5	250.0
D-81 (8)	4′-(4,6-diamino-1,2-dihydro-2,2-dimethyl-1-*s*-triazinyl)-phenyl	"	methyl	375000	2250000
D-82	2′-bromophenyl	methyl	methyl	164000	1520000
D-83	4′-tolyl	"	"	40.0	340.0
D-87	4′-chlorophenyl	"	*n*-propyl	400	1800

(1) $R_4 = H$ except in the case of compound D-32 • HCl where $R_4 = CH_3$.
(2) Free base microbiologically active.
(3) Dihydrochloride microbiologically active.
(4) Nitrite, nitrate and bicarbonate microbiologically active.
(5) Picrate microbiologically active.
(6) Inactive un-blocked hydrochloride (see text).
(7) Sample received through the courtesy of Imperial Chemical Industries, Ltd., Manchester, England showed similar microbiological activity.
(8) As the dihydrochloride.

rectly in a Lumetron colorimeter adjusted so that medium blanks read zero versus the 650 mμ filter.

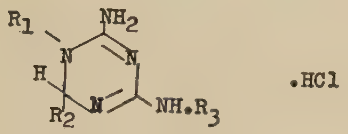
The test compounds were titrated from 1000 γ/ml or more to the minimal inhibiting dose in repeated serial assays in the presence of 0.01 and 0.001 γ/ml of PGA, amounts which represent excess and minimal concentrations supporting maximal growth under these experimental conditions. PGA and medium controls were included in each experiment. Assays in which low values in the PGA controls or growth in the medium controls occurred were discarded. The 50% minimal inhibiting dose[§] (10) for the excess and minimal concentrations of PGA was interpolated

---

[§] The 50% minimal inhibiting dose and inhibition index are relative values and do not necessarily bear any direct relationship to the minimal inhibiting dose required for the complete inhibition of a given inoculum in a given assay system. Although subject to somewhat less variation than "complete inhibition", variations in procedure and the basal medium are reflected in the mathematical interpolation of the 50% inhibition index, hence such values may not be exactly reproducible under other experimental conditions. However, the relative relationships among a series of inhibitors should be comparable at these 50% values irrespective of variation in assay systems.

TABLE II. Inhibition of *Streptococcus faecalis* #8043-PGA Assay Systems by Aldehyde Derivatives of Dihydrotriazine Series.

Structure (1)



Compound	R_1	R_2	Inhibition index vs. PGA .01 γ/ml	.001 γ/ml
D-31	phenyl	*n*-propyl	1500	2500
D-34 (2)	"	phenyl	2500	6200
D-37	4′-chlorophenyl	"	380	880
D-45 (3)	"	methyl	220	750
D-47	"	2′-chlorophenyl	550	750
D-48	"	*n*-propyl	235	1750
D-49	4′-anisyl	"	480	4800
D-55	3′,4′-dichlorophenyl	phenyl	62	440
D-57	2′,4′-dichlorophenyl	"	13000	110000
D-58	2′,5′-dichlorophenyl	"	7500	75000
D-59	4′-chlorophenyl	*i*-propyl	1050	4400
D-60 (2,3)	"	ethyl	200	500
D-62	3′,4′-dichlorophenyl	*i*-propyl	365	990
D-63 (3)	"	ethyl	40	104
D-67	"	methyl	32.5	125
D-76	"	*n*-propyl	95	350
D-84	4′-chlorophenyl	*n*-hexyl	45	220
D-86 (1)	"	phenyl	4200	6200
D-88	2′-tolyl	*n*-propyl	640000	200000

(1) $R_3 = H$ except in compound D-86 • HCl where $R_3 = CH_3$.
(2) Free base also microbiologically active.
(3) Dihydrochloride also microbiologically active.

from a plot of the data obtained from such titrations.

Inhibition analyses(11) were done in systems containing 0.01 γ/ml of PGA, to which were added the minimal concentrations of the respective dihydrotriazines which completely inhibited growth as determined by previous titration in the presence of excess concentrations of PGA. Various metabolites were then titrated in these inhibited systems to determine their ability to reverse dihydrotriazine inhibition. Those derivatives characterized by a 50% inhibition index of less than 100 were studied under these experimental conditions. Similar assays inhibited with 4-aminopteroylglutamic acid (4-APGA) also were done for purposes of comparison.

Results. The 50% inhibition indices obtained with 2,2-di-substituted and 2-mono-substituted derivatives are presented in Tables I and II. Microbiological activity varies considerably with substitution in the molecule in either series. Maximum activity is obtained with 2,2-dimethyl substitutions, *e.g.*, D-20 • HCl ($R_2 = R_3 = CH_3$) exhibits greater activity than its isomeric derivative D-60 • HCl ($R_2 = C_2H_5$) (Table II) or D-43 • HCl ($R_2 = CH_3$, $R_3 = C_2H_5$) (Table I). If the structure is otherwise unaltered microbiological activity is enhanced by *para*-or-*meta*-halogen substitution in the phenyl ring and activity is further increased by 3′,4′-di-halogen-substitution, as illustrated by a comparison of the microbiological activity of compounds D-23 • HCl, D-20 • HCl, D-69 • HCl, D-65 • HCl, and D-54 • HCl[‖] (Table I).

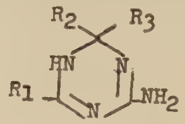
Substitution of a sufficiently large group at the *ortho*-position of the phenyl ring (*e.g.*, Cl in compound D-68 • HCl) results in reduced microbiological activity and similarly, the presence of a large blocking group at the 2-position of the triazine ring (*e.g.*, penta-

---

[‖] Two samples of this compound were used in these studies, one synthesized in these laboratories by Dr. E. J. Modest and another supplied through the courtesy of Imperial Chemical Industries, Ltd., Manchester, England.

TABLE III. Relative Inactivity of Isomers of Certain Dihydrotriazines in *Streptococcus faecalis* #8043-PGA Assay Systems.

Structure (1)



Compound	R_1	R_2	R_3	Inhibition index vs. PGA	
				.01 γ/ml	.001 γ/ml
DX-19	4′-carbethoxyanilino	methyl	methyl	140000	980000
DX-20	4′-chloroanilino	"	"	6400	52000
DX-20 • HNO_3	"	"	"	32500	325000
DX-20 • HCl	"	"	"	56000	375000
DX-20 • 2HCl	"	"	"	5800	35000
DX-22	4′-carboxyanilino	"	"	188000	1130000
DX-23	anilino	"	"	600000	6000000
DX-29 (1)	"	"	"	75000	750000
DX-30 • HCl	N-methylanilino	"	"	200000	2000000
DX-34	anilino	H	phenyl	156000	1560000
DX-35 • HCl (1)	4′-chloroanilino	methyl	methyl	20000	200000
DX-60	"	H	ethyl	49000	500000

(1) In compound DX-35, NH_2 is replaced by $NHCH_3$; in compound DX-29, NH_2 is replaced by $N(CH_3)_2$.

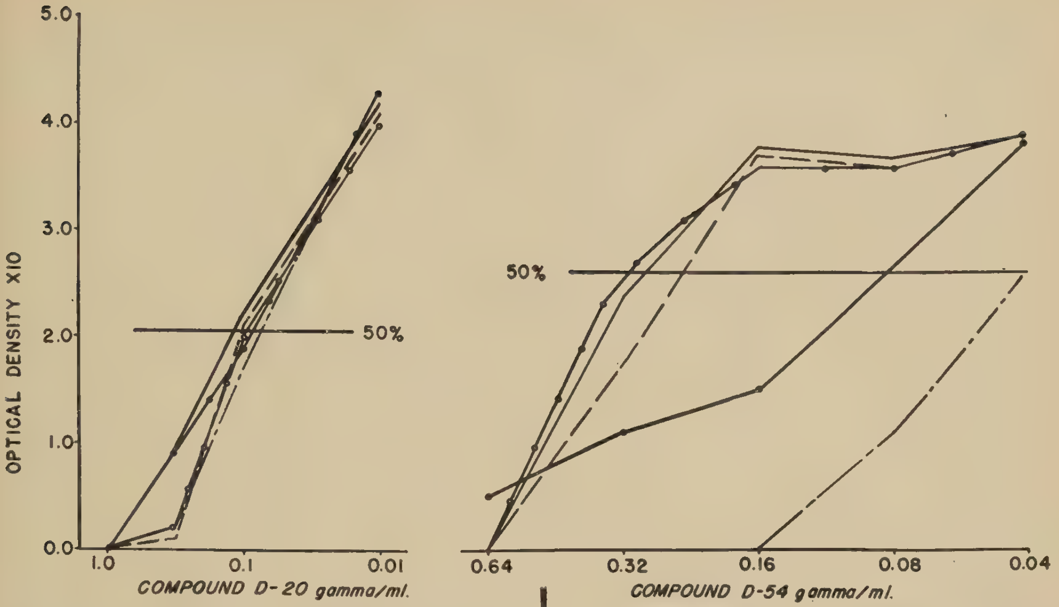
methylene in compound D-40 • HCl) also diminishes microbiological activity (Table I). All hydrochlorides studied thus far which do not possess either of these structural features have exhibited microbiological activity except the *para*-carboxy derivative D-22 • HCl (Table I).

The free bases prepared by careful neutralization of the corresponding dihydrotriazine hydrochlorides also exhibit microbiological activity (Tables I, II) while the isomeric (DX) compounds prepared by rearrangement of the corresponding (D • HCl) structures under appropriate conditions(1,2,5) are relatively inert in *Streptococcus faecalis*-PGA systems (Table III). A more detailed study of the relation of chemical structure to microbiological activity, as well as the reduced activity resulting from substitution at the *ortho*-position of the phenyl ring and the 2-position of the triazine ring will be presented in subsequent reports.

Inspection of the 50% inhibition indices (Tables I, II) indicates that these dihydrotriazines exhibit a non-competitive antagonism(12). The 50% inhibition indices computed over a wide range of concentrations of PGA (*e.g.*, Fig. 1) further illustrate the minimal effect of increasing concentrations of PGA on the 50% minimal inhibiting dose. The results of other experiments in which excesses of PGA were added to the basal medium containing 0.01 γ/ml of PGA and inhibitory concentrations of dihydrotriazine indicate that if at all, inhibition is reversible by PGA only within a narrow range of concentration of inhibitor. The magnitude of this range varies somewhat with different derivatives but in each instance concentrations of dihydrotriazine slightly in excess of the minimal inhibiting doses are uniformly irreversible by excess PGA.

The results of inhibition analyses with the 2,2-di-substituted derivatives exhibiting most activity versus PGA (D-20 • HCl, D-23 • HCl, D-54 • HCl) and a derivative of moderate activity (D-19 • HCl) together with the most active (D-63 • HCl) and least active (D-76 • HCl) 2-mono-substituted derivatives are summarized in Table IV. The inhibition induced by all derivatives was reversible by dihydropteroylglutamic acid¶ (dihydro-PGA)(13), N^{10}-formylpteroylglutamic acid¶ (N^{10}-formyl-PGA)(14), leucovorin¶(15), and citrovorum factor¶ (CF)(16) but not by PGA. The concentrations of the various metabolites required for the reversal of different derivatives varied but did not appear to be directly related to the 50% inhibition index vs PGA.

¶ We wish to express our appreciation to the Lederle Laboratories, Pearl River, N. Y., for generous samples of these compounds.



PTEROYLGLUTAMIC ACID gamma/ml.		50% INHIBITION gamma/ml.	INHIBITION INDEX
10.0	— — — —	0.10	0.01
1.0	———	0.10	0.10
0.1	—•—•—•	0.095	0.95
0.01	—•—•—•	0.095	9.50
0.001	—·—·—	0.095	95.0

PTEROYLGLUTAMIC ACID gamma/ml.		50% INHIBITION gamma/ml.	INHIBITION INDEX
100.0	—•—•—•	0.31	0.0031
10.0	— — — —	0.28	0.028
1.0	———	0.30	0.30
0.01	—•—•—•	0.10	10.0
0.001	—·—·—	0.035	35.0

FIG. 1.
Effect of increasing concentrations of PGA on 50% minimal inhibiting dose of dihydrotriazine.

Similar studies with the purines, pyrimidines, nucleotides and nucleosides indicate that unlike 4-APGA(17) the dihydrotriazines are not reversed by excesses of adenine and guanine or their derivatives but are partially reversed by excess uracil or uridylic acid, desoxyribonucleic acid and more completely by thymine (Table IV).

In general, excess concentrations of other vitamins have no effect on the inhibition of *Streptococcus faecalis* by these dihydrotriazines. However, certain derivatives of this series exhibit considerable microbiological activity versus *Lactobacillus arabinosus* No. 17-5** in niacin assay systems(1). The inhibition of *Streptococcus faecalis* by such compounds is partially reversed by high concentrations of niacin; for example, D-54 • HCl in concentrations circa 1.0 γ/ml inhibits *Lactobacillus arabinosus* in niacin assay systems, while concentrations circa 100 γ/ml of niacin partially reverse D-54 • HCl inhibition in *Streptococcus faecalis*-PGA assay systems. Detailed studies of the inhibition observed in *Lactobacillus arabinosus* and other *Lactobacillus* assay systems will be reported elsewhere.

Discussion. The microbiological activity of these symmetrical dihydrotriazines in *Streptococcus faecalis* systems differs from that exhibited by structural analogs of PGA in that inhibition is essentially non-competitive over a wide range of concentrations of PGA and is irreversible by excess PGA. Subsequent to the original communication from these laboratories(1) several classes of compounds bearing a formal structural relationship to these dihydrotriazines, namely aryl-diamino-pyrimidines(18,19),-*as*-triazines(19) and -guanazoles(19) also have been reported to be non-competitive inhibitors of PGA in *Streptococcus faecalis* systems. Consideration of the biologically active configuration of the sym-

---

** *Lactobacillus plantarum,* A.T.C.C. No. 8014.

TABLE IV. Reversal of Dihydrotriazine Inhibition in *Streptococcus faecalis* #8043-PGA Assay Systems.

	Concentration in γ/ml reversing inhibition of:						
	D-19·HCl	D-20·HCl	D-23·HCl	D-54·HCl	D-63·HCl	D-76·HCl	4-APGA
	γ/ml						
Metabolite	1.25	.60	.60	.3	2.0	4.0	.1
Pteroylglutamic acids							
Pteroylglutamic acid	.0	.0	.0	.0	.0	.0	*.1*
Dihydropteroylglutamic acid	.01	1.0	1.0	10.0	1.0	1.0	.1
N^{10}-formylpteroylglutamic acid	.01	1.0	20.0	20.0	1.0	1.0	.1
Leucovorin	.00001	.0001	.0001	.0001	.0001	.00001	.000005
Citrovorum factor	.0000005	.00005	.00005	.00005	.0005	.00005	.00005
Purines*							
Adenine	.0	.0	.0	.0	.0	.0	*100.0‡*
Guanine	.0	.0	.0	.0	.0	.0	*100.0‡*
Pyrimidines*							
Thymine	1.0	1.0	10.0	10.0	1.0	1.0	10.0
Uracil	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Cytosine	.0	.0	.0	.0	.0	.0	.0
Nucleotides*†							
Adenylic acid (yeast)	.0	.0	.0	.0	.0	.0	*500.0‡*
Guanylic acid	.0	.0	.0	.0	.0	.0	*500.0‡*
Cytidylic acid	.0	.0	.0	.0	.0	.0	.0
Uridylic acid‡	500.0	500.0	500.0	500.0	500.0	500.0	1000.0
Nucleosides*†							
Adenosine	.0	.0	.0	.0	.0	.0	*1000.0‡*
Guanosine	.0	.0	.0	.0	.0	.0	*1000.0‡*
Cytidine	.0	.0	.0	.0	.0	.0	.0
Uridine	.0	.0	.0	.0	.0	.0	.0
Nucleic acids*							
Ribose	.0	.0	.0	.0	.0	.0	.0
Desoxyribose‡	100.0	100.0	100.0	100.0	100.0	100.0	100.0

* Tested in concentrations 100-.1 γ/ml.

† Thymidylic acid and thymidine not tested.

‡ Circa 50—75% reversal.

Addition of PABA to 100 γ/ml had no sparing effect in these systems.

Similar results were obtained with sample of dichloro-derivative (D-54·HCl) supplied by Imperial Chemical Industries, Ltd.

metrical dihydrotriazines in relation to these other types of compounds will be reported elsewhere.

It has become generally accepted that CF is the physiologically active form of PGA as suggested by Sauberlich(20) and that PGA is concerned with the utilization of thymine or thymidine in the synthesis of nucleic acids (21-24). CF(25,26) and thymidine(23,26) have been shown to replace PGA and to reverse inhibition produced by analogs of PGA in microbiological systems. These and other microbiological studies, toxicity studies in mice(25,26) and more recent *in vitro* observations on liver slices(27) suggest that the inhibition produced by analogs of PGA is due at least in part to interference with the conversion of PGA to CF in biological systems.

The ability of reduced PGA and formylated PGA as well as CF and thymine to reverse dihydrotriazine inhibition suggests that like 4-APGA, the dihydrotriazines may interfere with the conversion of PGA to CF. However, the dihydrotriazines differ from 4-APGA in that non-competitive inhibition by the former is not relieved by adenine or guanine and is not reversed by PGA. In this respect, the behavior of the dihydrotriazines in *Streptococcus faecalis* systems resembles that of 4-APGA and other analogs of PGA in mammals in that once inhibition (or toxicity) has occurred, it is not readily reversed by PGA

(28) but can be reversed with CF(25,26) and nucleic acids(17). Preliminary experiments indicate that the temporal sequence of exposure to inhibitor and metabolite may be of importance in the dihydrotriazine inhibition of *Streptococcus faecalis* as has been observed with 4-APGA in mammals(29).

Summary. A series of 71 *1,2*-dihydro-*s*-triazines have been studied in *Streptococcus faecalis* No. 8043-pteroylglutamic acid assay systems. The active derivatives exhibit noncompetitive inhibition over a wide range of concentrations of PGA and differ from 4-aminopteroylglutamic acid in that inhibition is not relieved by adenine or guanine and is irreversible with pteroylglutamic acid. Differences in microbiological activity could be correlated with certain variations in structure and substitution in the molecule with the series. Maximum activity is obtained with 2,2-dimethyl substitution in the triazine ring together with *para*- and *meta*-halogen substituents in the phenyl ring. The inhibitory effect of these compounds on *Streptococcus faecalis* No. 8043 is reversed by appropriate concentrations of dihydropteroylglutamic acid, N^{10}-formylpteroylglutamic acid, synthetic and natural citrovorum factor and thymine, suggesting that these compounds interfere with the conversion of pteroylglutamic acid to citrovorum factor.

The invaluable technical assistance of Brittmarie Lindsjo, Carolyn Hall, Janet Smith and Edward Botan during the course of these studies is gratefully acknowledged.

---

1. Modest, E. J., Foley, G. E., Pechet, M. M., and Farber, S., *J. Am. Chem. Soc.*, 1952, v74, 855.

2. Modest, E. J., Abst. of Papers, 122nd Meeting Am. Chem. Soc., 1952, 9L, Sept. 16, Atlantic City, N. J.

3. Farber, S., Diamond, I., Foley, G. E., and Modest, E. J., (Abst.), *Am. J. Path.*, 1952, v28, 559.

4. Foley, G. E., and Modest, E. J., (Abst.), Proc. 52nd General Meeting, Soc. Am. Bact., 1952, 63, April 27-May 1, Boston, Mass.

5. Modest, E. J., in press, 1953.

6. Carrington, H. C., Crowther, A. F., Davey, D. G., Levi, A. A., and Rose, F. L., *Nature* (Lond.), 1951, v168, 1080.

7. Crowther, A. F., and Levi, A. A., *Brit. J. Pharmacol.*, 1953, v8, 93.

8. Assn. of Vitamin Chemists, Inc., *Methods of Vitamin Assay*, 1951; ed. 2, Interscience Publishers Inc., New York.

9. Teply, L. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1945, v157, 303.

10. Hutchings, B. L., Mowat, J. H., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and Subbarow, Y., *J. Biol. Chem.*, 1947, v170, 323.

11. Shive, W., and Roberts, E. C., *J. Biol. Chem.*, 1946, v162, 463.

12. Woolley, D. W., *A Study of Antimetabolites,* 1952, ed. 1, J. Wiley and Sons, Inc., New York.

13. O'Dell, B. L., Vandenbelt, J. M., Bloom, E. S., and Pfiffner, J. J., *J. Am. Chem. Soc.*, 1947, v69, 250.

14. Gordon, M., Ravel, J. M., Eakin, R. E., and Shive, W., *J. Am. Chem. Soc.*, 1948, v70, 878.

15. Brockman, J. A., Jr., Roth, B., Broquist, H. P., Hultquist, M. E., Smith, J. M., Jr., Fahrenbach, M. J., Cosulich, D. B., Parker, R. P., Stokstad, E. L. R., and Jukes, T. H., *J. Am. Chem. Soc.*, 1950, v72, 4325.

16. Sauberlich, H. E., and Bauman, C. A., *J. Biol. Chem.*, 1948, v176, 165.

17. Skipper, H. E., Bell, M., and Chapman, J. B., *Cancer,* 1951, v4, 357.

18. Hitchings, G. H., Falco, E. A., VanderWerff, H., Russell, P. B., and Elion, G. B., *J. Biol. Chem*, 1952, v199, 43.

19. Hitchings, G. H., Maggiolo, A., Russell, P. B., VanderWerff, H., and Rollo, I. M., *J. Am. Chem. Soc.*, 1952, v74, 3200.

20. Sauberlich, H. E., *J. Biol. Chem.*, 1949, v181, 467.

21. Stokes, J. L., *J. Bact.*, 1944, v48, 201.

22. Shive, W., Eakin, R. E., Harding, W. B., Ravel, J. M., and Sutherland, G. L., *J. Am. Chem. Soc.*, 1948, v70, 2099.

23. Broquist, H. P., Stokstad, E. L. R., Hoffman, C. E., Belt, M., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 549.

24. Rogers, L. L., and Shive, W., *J. Biol. Chem*, 1948, v172, 751.

25. Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H, *Fed. Proc.*, 1950, v9, 18.

26. ———, *J. Biol. Chem.*, 1950, v185, 399.

27. Nichol, C. A., and Welch, A. D., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 403.

28. Philips, F. S., Thiersch, J. B., and Ferguson, F. C., *Ann. N. Y. Acad. Sci.*, 1950, v52, 1349.

29. Greenspan, E. M., Goldin, A., and Schoenbach, E. B., *Cancer*, 1950, v3, 856.

Received July 9, 1953. P.S.E.B.M., 1953, v83.

Chemical and Biological Studies on *1,2*-Dihydro-*s*-triazines. V. Inhibition of Derivatives of Pteroylglutamic Acid. (20478)

G. E. Foley. (Introduced by Harry Eagle)

From the Laboratories of Microbiology, The Children's Cancer Research Foundation and the Department of Pathology, Harvard Medical School, at Children's Medical Center, Boston, Mass.

Previous reports have described the non-competitive inhibition of pteroylglutamic acid (PGA) in *Streptococcus faecalis* No. 8043* systems by the dihydrotriazine hydrochlorides (1,2) and the reversal of this inhibition by dihydropteroylglutamic acid† (dihydro-PGA), N^{10}-formylpteroylglutamic acid† (N^{10}-formyl-PGA), leucovorin† and citrovorum factor† (CF)(3). The present report concerns the inhibition of these latter metabolites by appropriate concentrations of the dihydrotriazine hydrochlorides.

Methods. The media, methods of preparation and assay employed with *S. faecalis* were as described previously(2,3) except that minimal and excess concentrations of dihydro-PGA, N^{10}-formyl-PGA, leucovorin and CF were substituted for PGA in the respective assays. The medium described by Sauberlich and Bauman(4) was used for *Leuconostoc citrovorum* No. 8081‡ assays; otherwise methods were the same except that titrations were incubated for 48 hours. The dihydrotriazine hydrochlorides characterized by a 50% inhibition index of less than 100 vs PGA(3) were titrated in these systems and the 50% inhibition indices(5) computed.

Results. The calculated 50% inhibition indices of representative compounds vs these metabolites are compared with those obtained vs PGA in *S. faecalis* and vs CF in *L. citrovorum* assay systems in Table I. The inhibition of reduced or formylated PGA as well as CF could be accomplished in *S. faecalis* systems only by considerably higher inhibitor: metabolite ratios than those which were effective vs PGA. The 2,2-dimethyl-substituted derivatives in general were more active than the analogous 2-mono-substituted compounds. The unsubstituted phenyl (D-23 • HCl) and 3′-chlorophenyl (D-69 • HCl) compounds exhibited greatest activity vs CF in both microbiological systems. Halogen substitution at the 4′- and 3′,4′-positions of the phenyl ring had little effect on activity vs dihydro-PGA or N^{10}-formyl-PGA but resulted in decreased activity vs CF in either system as illustrated by a comparison of the activity of compounds D-23 • HCl, D-20 • HCl, and D-54 • HCl§ while methyl substitution at the *4*′-position of the phenyl ring (D-83 • HCl) resulted in a marked decrease in activity vs dihydro-PGA as well as CF (Table I). These relationships between structure and biological activity vs these metabolites will be considered elsewhere in greater detail.

The inhibition indices obtained with a number of derivatives in preliminary titrations suggested competitive inhibition. However, when such compounds were titrated vs a wider range of concentration the inhibition indices shifted with increasing concentrations of metabolites, indicating that competitive inhibition obtains only over a relatively limited range of metabolite concentrations (Table II). This range varied with different derivatives, but in no instance did it exceed a 5-10 fold difference in concentration. The inhibition of CF in *L. citrovorum* assay systems appeared to be non-competitive over the range of metabolite concentrations noted in Table II.

Discussion. The inhibitory activity of this series of dihydrotriazines vs dihydro-PGA, N^{10}-formyl-PGA and CF resembles that exhibited vs PGA(3) in that a competitive in-

---

* A.T.C.C. No. 8043.

† We wish to thank the Lederle Laboratories, Pearl River, N. Y., for generous samples of these compounds.

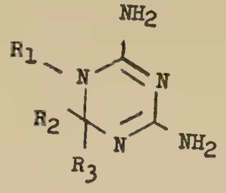
Another sample of dihydro-PGA synthesized in these laboratories by Dr. E. J. Modest also was used in these studies.

‡ A.T.C.C. No. 8081.

§ Two samples of this compound were used in these studies, one synthesized in these laboratories by Dr. E. J. Modest and another supplied through the courtesy of Imperial Chemical Industries, Ltd., Manchester, England.

TABLE I. Comparative Activity of Dihydrotriazine Hydrochlorides vs. PGA, Dihydro-PGA, N^{10}-Formyl-PGA and CF.

Structure



				50% Inhibition index				
				Strep. faecalis #8043				*Leuc. citrovorum* #8081
Compound	R_1	R_2	R_3	PGA* .01	Dihydro-PGA .001	N^{10}-formyl-PGA .001	CF† .001	CF† .001
D-23 • HCl	phenyl	methyl	methyl	10	800	820	2500	9200
D-69 • HCl	3′-chlorophenyl	”	”	32.5	3000	860	2000	9370
D-83 • HCl	4′-methylphenyl	”	”	40	22000	860	114000	180000
D-20 • HCl	4′-chlorophenyl	”	”	9.5	800	740	10500	20000
D-84 • HCl	”	H	*n*-hexyl	45	15000	3600	48000	116000
D-54 • HCl‡	3′,4′-chlorophenyl	methyl	methyl	10	760	390	11600	22000
D-75 • HCl	”	”	ethyl	37.5	2000	1800	200000	187000
D-76 • HCl	”	H	*n*-propyl	95.0	60000	6200	200000	184000

Concentration of metabolites in γ/ml. * See bibliography #1 and 2. † Similar results with leucovorin. ‡ Similar results with sample supplied by Imperial Chemical Industries, Ltd.

TABLE II. Inhibition Indices of Dihydrotriazine Hydrochlorides vs. Increasing Concentrations of Metabolites.

Compound	50% Inhibition index *Streptococcus faecalis* #8043			
	Dihydro-PGA, γ/ml			
	.001	.01	.1	1.0
D-20 • HCl	800	1200	100	12
D-84 • HCl	1100	460	55	5
	N^{10}-formyl-PGA, γ/ml			
	.0001	.001	.01	.1
D-23 • HCl	920	820	340	42
D-69 • HCl	550	860	400	42
	Citrovorum factor, γ/ml*			
	.00005	.0005	.001	.002
D-69 • HCl	2000	10800	9370	18750
D-84 • HCl	76000	52000	48000	25000

* Similar results with leucovorin.

hibitor: metabolite relationship obtained only over a relatively narrow range of metabolite concentrations. Although this range was broader than that observed vs PGA, increasing concentrations of dihydro-PGA, N^{10}-formyl-PGA, and CF resulted in a marked shift in the inhibition index. Similar changes in the inhibition index with increasing concentrations of metabolite have been observed with 4-aminopteroylglutamic acid(6). These dihydrotriazines then are not typical examples of competitive inhibitors according to classical definition(7); thus the present results are interpreted to indicate that the inhibition of dihydro-PGA, N^{10}-formyl-PGA and CF by these dihydrotriazines is essentially non-competitive.

The decreased effectiveness of the inhibition of dihydro-PGA and N^{10}-formyl-PGA as compared to PGA is further indication that these compounds interfere with the biological conversion of PGA to CF in *S. faecalis* systems. The similarity in the response of either CF system to the compounds in this series suggests that the mechanism of utilization and inhibition of CF may the same in *S. faecalis* and *L. citrovorum*. However, the marked increase in the inhibition indices vs CF as compared to PGA, dihydro-PGA and N^{10}-formyl-PGA suggests that the inhibition of CF systems may involve a different mechanism of action, *i.e.*, interference with the utilization of CF rather than inhibition of the synthesis of

CF in PGA systems. This possible difference in mechanism of action may be of significance in the interpretation of the effects of the dihydrotriazines in other biological systems.

Studies with several of the early compounds in this series failed to demonstrate microbiological activity in systems in which thymine was substituted for PGA(1). However, experiments now in progress indicate that a number of derivatives synthesized more recently in these laboratories may exhibit significant anti-thymine activity. Details must await completion of current experiments.

Summary. Dihydropteroylglutamic acid, N^{10}-formylpteroylglutamic acid and citrovorum factor are inhibited non-competitively by a number of *1,2*-dihydro-*s*-triazines when substituted for pteroylglutamic acid in *S. faecalis* No. 8043 assay systems, but only by inhibitor:metabolite ratios considerably higher than those required for the inhibition of pteroylglutamic acid in the same microbiological system. Citrovorum factor also is similarly inhibited in *L. citrovorum* No. 8081 assay systems. The phenyl- and 3′- and 4′-halophenyl-2,2-dimethyl derivatives were the most active compounds of the series and certain correlations can be made between structure and microbiological activity vs these metabolites.

---

1. Modest, E. J., Foley, G. E., Pechet, M. M., and Farber, S., *J. Am. Chem. Soc.*, 1952, v74, 855.
2. Foley, G. E., and Modest, E. J., Proc. 52nd Gen. Meeting, Soc. Amer. Bact., 1952, 63, April 27-May 1, Boston, Mass.
3. Foley, G. E., Proc. Soc. Exp. Biol. and Med., 1953, v83, 57.
4. Sauberlich, H. E., and Bauman, C. A., *J. Biol. Chem.*, 1948, v176, 165.
5. Hutchings, B. L., Mowat, J. H., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and Subbarow, Y., *J. Biol. Chem.*, 1947, v170, 323.
6. Oleson, J. J., Hutchings, B. L., and Subbarow, Y., *J. Biol. Chem.*, 1948, v175, 359.
7. Woolley, D. W., *A Study of Antimetabolites*, 1952, ed. 1, J. Wiley and Sons, Inc., New York.

Received July 9, 1953. P.S.E.B.M., 1953, v83.

Chemical and Biological Studies on *1,2*-Dihydro-*s*-Triazines. VI. Development of Resistance by *Streptococcus faecalis* No. 8043. (20479)

G. E. Foley and P. L. Watson. (Introduced by Harry Eagle.)

From the Laboratories of Microbiology, The Children's Cancer Research Foundation and the Department of Pathology, Harvard Medical School at the Children's Medical Center, Boston, Mass.

The development of resistance to various analogs of pteroylglutamic acid (PGA) has been described in human leukemia(1) and in experimental mouse leukemia(2-8). Resistance to these chemotherapeutic agents also has been reported as occurring in *Tetrahymena geleii* by Kidder, Dewey, and Parks(9), in *Streptococcus faecalis* No. 8043 by Foley(10), and more recently by Burchenal, Waring, and Hutchison(11).

Previous publications from these laboratories have described the non-competitive inhibition of *S. faecalis* No. 8043* in PGA assay systems by another class of antagonists, the *1,2*-dihydro-*s*-triazines(12,13). The purpose of the present report is to describe experiments dealing with the isolation and origin of a number of strains of *S. faecalis* No. 8043 which are resistant to inhibition in PGA assay systems by these compounds. Media, methods of preparation and assay were identical with those previously described(12,13) unless otherwise stated.

Isolation of resistant strains. When *S. faecalis*-PGA assay systems containing inhibiting concentrations of the dihydrotriazine hydrochlorides were incubated beyond the usual 18-24 hour period, growth occurred after a prolonged lag phase. Thus the minimal inhibiting concentration of 4,6-diamino-l-(3′,4′-dichlorophenyl)-1,2-dihydro-2,2-dimeth-

---

* A.T.T.C. No. 8043.

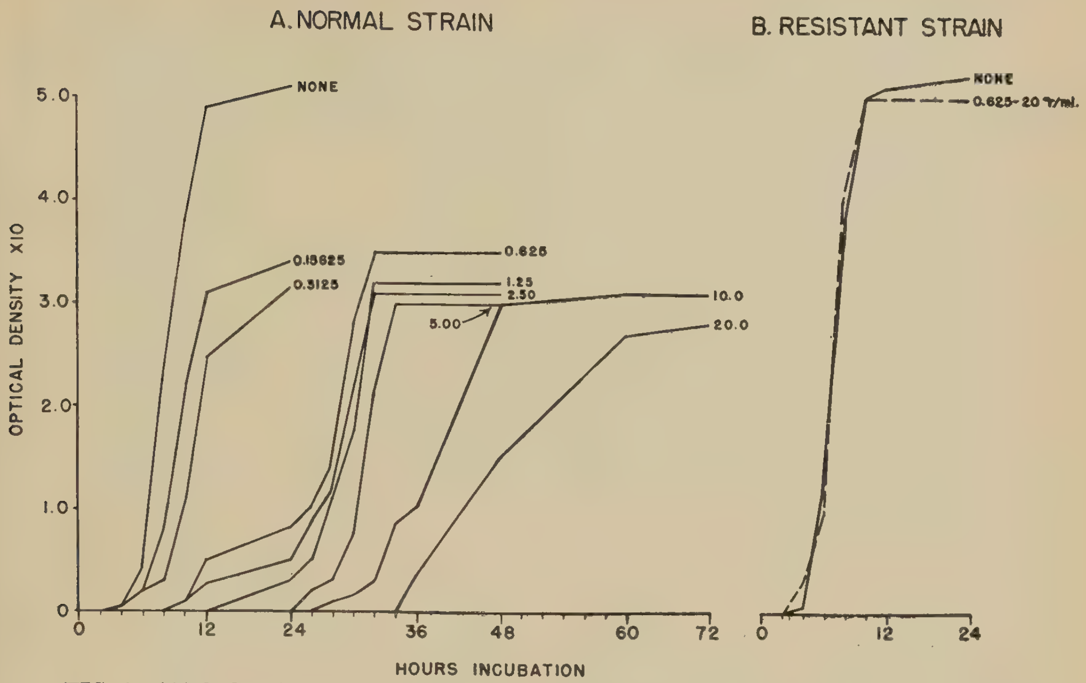


FIG. 1. (A) Delayed growth of *Streptococcus faecalis* #8043 in PGA assay media containing 0.01 γ PGA and stated concentrations of D-54 • HCl/ml. (B) Growth of D-54 • HCl-resistant strains in same medium with and without D-54 • HCl.

yl-*s*-triazine hydrochloride† (D-54 • HCl) was 0.625-1.25 γ/ml in systems containing 0.01-0.001 γ/ml PGA. However, delayed growth occurred within 72 hours in the presence of all concentrations through 20 γ/ml (Fig. 1A). No measurable growth occurred even after 108 hours incubation in the presence of 40 or more γ/ml of D-54 • HCl. When cultures obtained in this manner were exposed to 0.625-20 γ/ml of D-54 • HCl all exhibited increased resistance, the degree of resistance in each instance corresponding roughly to the concentration to which each strain had been previously exposed. All strains exhibited a normal growth curve in the presence of its corresponding concentration of D-54 • HCl (Fig. 1B).

The original assay tubes containing concentrations of more than 20 γ/ml of D-54 • HCl in which no growth had occurred after 108 hours were concentrated in the centrifuge and cultured on blood agar and PGA assay media without D-54 • HCl. Viable *S. faecalis* were recovered from assay tubes containing 40-640 γ/ml but not from those containing 1280 and 2560 γ/ml D-54 • HCl. Those strains recovered from the assay tubes containing 40-640 γ/ml were inhibited by 0.625 γ/ml of D-54 • HCl.‡ The delayed growth of cells resistant to 0.625-20 γ/ml D-54 • HCl in these experiments indicates that stock cultures of *S. faecalis* may contain cells which vary in sensitivity to the inhibitor over this entire range of concentration. The number of resistant cells probably decreases with increas-

---

† Two samples of this compound were used in these studies, one synthesized in these laboratories by Dr. E. J. Modest and another supplied through the courtesy of Imperial Chemical Industries, Ltd., Manchester, England.

‡ A single colony originally exposed to 40 γ/ml was found to be resistant to 10 γ/ml D-54•HCl, suggesting in this instance the chance occurrence of cells resistant to this concentration in the original inoculum or the appearance of resistance as the result of chance cell division which occurred in the inhibited culture. Repeated attempts to duplicate this observation have been unsuccessful; resistant cells have not appeared in assays containing more than 20-25 γ/ml D-54•HCl.

TABLE I. Survival of *Streptococcus faecalis* #8043 in PGA Media* Containing D-54 • HCl.

D-54 • HCl, γ/ml	Expected survivors† (most probable No.)	Observed survivors, pour plates	Survivors per 1 × 10^6 cells	Mean frequency‡
.625	127	200	.027	1 in 4.82 × 10^7
1.25	57	90	.012	1 " 1.07 × 10^8
2.50	21	30	.004	1 " 3.00 × 10^8
5.00	—	10	.0013	1 " 7.50 × 10^8
10.0	—	5	.00065	1 " 1.50 × 10^9
20.0	—	5	.00065	1 " 1.50 × 10^9
40.0-2560	—	0	0	

* PGA, .01 γ/ml.

† The disparity between expected and observed survivors is due to differences in the ability of these broth and agar media to initiate growth from a single unit, *i.e.,* a pair or a chain of cells of *Streptococcus faecalis.*

‡ In standard suspensions of 7.5 × 10^9 cells/ml.

ing concentrations, as evidenced by the progressively longer lag phase. The isolation of these resistant strains could be explained on the basis of simple selection or alternately by adaptation by a few cells to these concentrations of D-54 • HCl.

The frequency of cells resistant to increasing concentrations of D-54 • HCl in standard inocula prepared from the stock strain of *S. faecalis* was determined by dilution experiments(14) and by actual count of the survivors at increasing concentrations of inhibitor in agar pour plates. The number of survivors observed at increasing concentrations of inhibitor in agar checks reasonably well with the expected number as estimated from dilution data and the frequency of resistant cells decreases with increasing concentrations of D-54 • HCl (Table I). Representative samples of the surviving colonies were fished from these agar plates to PGA assay broth containing 0.625 γ/ml D-54 • HCl, incubated and studied in duplicate serial assays against D-54 • HCl. All but one of the 60 strains so studied failed to exhibit resistance to concentrations of inhibitor significantly greater than that to which it had been originally exposed. The single exception, a colony fished from agar containing 0.625 γ/ml D-54 • HCl required 48 hours to grow in subculture and when assayed was found to be resistant to 500 γ/ml D-54 • HCl.

Resistant strains isolated from the stock culture in this manner exhibited increasing resistance when carried in subculture in the presence of increasing concentrations of inhibitor, the resistance of the survivors increasing *pari passu* to the concentration to which the strains had been exposed. By serial passage in this manner, resistance was increased progressively and a culture completely resistant to 1000 γ/ml of D-54 • HCl was obtained after 14 serial passages (Fig. 2). Data on the frequency of resistant cells in the stock culture (Table I) indicate that it is unlikely that cells exhibiting this degree of resistance were contained in the original inocula. This order of resistance therefore must have appeared as the consequence of the selective growth of cells in the original inocula which either by serial mutation or adaptation became progressively more resistant to the inhibitor. The apparently step-wise appearance of increasing levels of resistance (Fig. 2) actually reflects the level of resistance attained at the fortuitous time of sampling and the development of resistance probably does

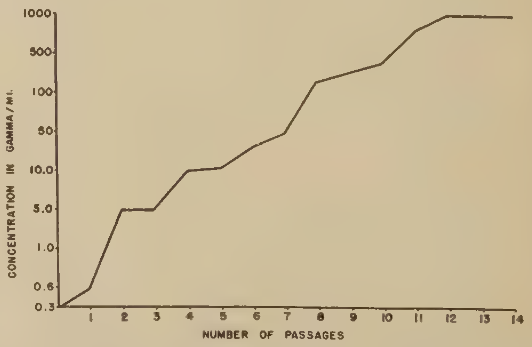


FIG. 2. Development of resistance in *Streptococcus faecalis* #8043 by serial passage in PGA assay media containing D-54 • HCl.

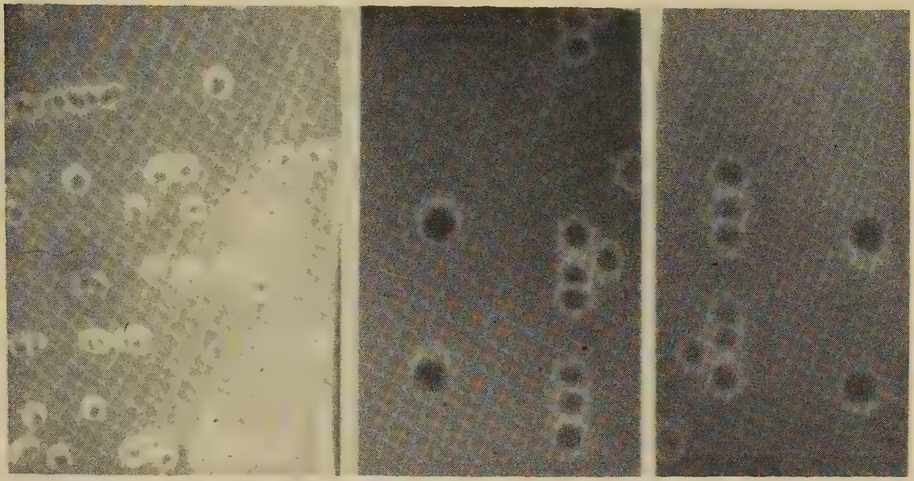


FIG. 3. Colony morphology of dihydrotriazine resistant variants: comparison with stock culture of *Streptococcus faecalis* #8043 (right). 24 hr culture on 5% horse blood agar, ×1.5.

not proceed in steps of these magnitudes.

These dihydrotriazine-resistant mutants have been carried through several generations in the laboratory in media with and without inhibitor with no significant loss of resistance during the past 9 months. Thus far the only recognizable difference between the mutant and parent strains other than their behavior with respect to inhibitors is in colony size, the resistant strains grow in uniformly smaller colonies which otherwise resemble those of the parent strains (Fig. 3).

Origin of resistance. Luria and Delbrück (15) developed a method of variance analysis to test the 2 hypotheses, mutation and adaptation generally postulated for the development of bacterial resistance. The appearance of dihydrotriazine resistance was analyzed by duplicate studies on 3 series of independent and replicate cultures derived from the stock culture. Standard inocula containing 7.5×10^9 cells were prepared from stock cultures and 0.1 ml of a 1×10^6 dilution (circa 750 cells) was inoculated into each of 21 tubes of PGA assay broth containing 0.01 γ/ml PGA. After 24 hours incubation at 37°C, 0.1 ml samples of each of 20 cultures was plated in PGA assay agar containing 1.25 γ/ml D-54 • HCl. A series of 15 replicate plates were prepared from the remaining culture in the same manner. After incubation at 37°C for 24 hours, the surviving colonies were enumerated in the usual manner. The results of these experiments are summarized in Table II.

Statistical analyses indicate that variation in the frequency of resistant cells appearing in independent cultures initiated from minimal inocula are greater than the chance variations observed in replicate plates of the same parent culture. These results are compatible with the hypothesis that resistance to D-54 • HCl appears as the result of spontaneous mutations(15).

Specificity of resistance. Resistant strains derived from these experiments with D-54 • HCl exhibited cross-resistance against a number of related aldehyde and ketone derivatives of the dihydrotriazine series, suggesting that resistance is related to the basic molecular configuration rather than to any of the particular substitutions mentioned (Table III). However, the D-54 • HCl resistant mutants so far studied have exhibited no cross-resistance with certain chloro- and dichloro-substituted 2,4-diamino-pyrimidines§ which also exhibit potent anti-PGA activity(16) and the PGA analogs 4-aminopteroylglutamic acid and 4-amino-N^{10}-methylpteroylglutamic acid (amethopterin).‖

---

§ These compounds were made available through the courtesy of Dr. G. H. Hitchings, the Wellcome Research Laboratories, Tuckahoe, N. Y.

‖ We wish to express our appreciation to the Lederle Laboratories, Pearl River, N. Y., for generous samples of these compounds.

TABLE II. Comparison of Frequency of Colonies of *Streptococcus faecalis* #8043 Resistant to 1.25 γ/ml D-54 · HCl in Independent and Replicate Cultures.

	Colony counts	
Culture	Individual cultures	Replicate cultures
1	16	7
2	5	8
3	0	10
4	10	9
5	80	10
6	6	18
7	59	9
8	10	7
9	16	10
10	20	11
11	17	19
12	0	5
13	11	15
14	12	7
15	32	7
16	37	
17	18	
18	72	
19	0	
20	30	
Mean	22.6	10.1
Variance	544.15	15.55
X^2	458.49	21.49
P	Much less than .001	.05

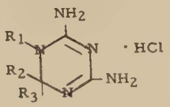
Such data represent a Poisson distribution; thus variance equals the mean. Chi square is defined as $\left(\frac{\text{observed} - \text{expected}}{\text{expected}}\right)^2$ and equals $\left(\frac{\text{observed} - \text{mean}}{\text{mean}}\right)^2$.

Discussion. Bacterial resistance to a number of inhibitors and lethal agents; bacteriophage(15), penicillin(17-19), sulfonamides (20), irradiation(21), and streptomycin(19) appears to originate from spontaneous mutations occurring in heterogeneous cell populations independently of the specific agents. Evidence for this view has been based largely upon results obtained with variance analyses as developed by Luria and Delbrück(15). Additional supportive evidence is afforded by the isolation of indirectly selected members of the populations such as those described by Burnet(22) and more recently by Lederberg and Lederberg(23) which exhibit specific resistance without previous exposure to the specific lethal agent or inhibitor. In the present studies, the results of variance analyses are compatible with the hypothesis that dihydrotriazine resistance also originates from spontaneous mutations which occur in the stock culture in the absence of inhibitor and which grow out selectively in its presence.

In the present experiments, a) the decreasing frequency of survivors at increasing concentrations of inhibitor, b) the correlation between the resistance of survivors and the concentrations of inhibitor to which they had been exposed, and c) the slow emergence of growth in cultures containing low concentrations of inhibitor suggest a specific adaptive process similar to that suggested by Eagle *et al.*(24) as a possible explanation for the development of slightly increased resistance to antibiotics on exposure to low concentrations. According to this interpretation, the varying degrees of resistance to minimal concentrations of inhibitor as exhibited by the stock culture may represent specific environmental adaptations occurring in susceptible cells. Sevag and Rosanoff(25) similarly concluded from their experiments with *Micrococcus pyogenes* var. *aureus* in complete and deficient media that resistance to streptomycin represents a specific adaptation by a sensitive cell. Gibson and Gibson(26) and more recently Eagle *et al.*(24) have suggested that the hypotheses of mutation and adaptation are not necessarily mutually exclusive, *i.e.*, within the spectrum of resistance of a "normal" culture the appearance of resistance to minimal concentrations of inhibitor may be adaptive, while the few highly resistant cells which appear represent spontaneous mutations.

The cross-resistance exhibited by these mutant strains with other derivatives of the dihydrotriazine series resembles the cross-resistance among the sulfonamides(27) and the analogs of PGA. Cross-resistance among the latter compounds has been observed in acute lymphoid mouse leukemia(2-8) and *T. geleii*(9) as well as in *S. faecalis*(11). The lack of significant cross-resistance with 4-APGA, a-methopterin, and the 2,4-diaminopyrimidines suggest an intimate relationship between resistance and the basic molecular configuration of the selective agent. However, studies with *E. coli*(21,28) and other

TABLE III. Cross-Resistance vs. Related Dihydrotriazines Exhibited by D-54 • HCl-Resistant Mutants of *Streptococcus faecalis* #8043.

Compound	 Structure R_1	R_2	R_3	Normal strain, 50% inhibition, γ/ml	Resistant strains, no inhibition at, γ/ml
D-19 • HCl	4′-carbethoxyphenyl	methyl	methyl	.375	1000
D-20 • HCl	4′-chlorophenyl	”	”	.095	1000
D-23 • HCl	phenyl	”	”	.10	1000
D-53 • HCl	2′,4′-dichlorophenyl	”	”	150.0	1000
D-55 • HCl	3′,4′-dichlorophenyl	H	phenyl	.62	100
D-56 • HCl	2′,5′-dichlorophenyl	methyl	methyl	3300.0	3000
D-57 • HCl	2′,4′-dichlorophenyl	H	phenyl	130.0	500
D-60 • HCl	4′-chlorophenyl	”	ethyl	2.0	1000
D-63 • HCl	3′,4′-dichlorophenyl	”	”	.4	100
D-65 • HCl	4′-bromophenyl	methyl	methyl	.12	1000
D-67 • HCl	3′,4′-dichlorophenyl	H	”	.325	100
D-68 • HCl	2′-chlorophenyl	methyl	”	141.0	1000
D-69 • HCl	3′-chlorophenyl	”	”	.325	100
D-76 • HCl	3′,4′-dichlorophenyl	H	*n*-propyl	.950	100
D-82 • HCl	2′-bromophenyl	methyl	methyl	1640.0	3000
D-54 • HCl	3′,4′-dichlorophenyl	”	”	.10	1000

All media contained .01 γ/ml PGA.

microorganisms(29) indicate that resistance to a number of apparently unrelated agents may appear following exposure to a single selective agent. Experiments are now in progress on the elucidation of the mechanism of dihydrotriazine resistance and on the continued search for mutants exhibiting cross-resistance with other PGA antagonists.

Summary. A number of strains have been isolated from stock cultures of *S. faecalis* No. 8043 which are resistant to inhibition by the *1,2*-dihydro-*s*-triazines in pteroylglutamic acid assay systems. The strains studied so far exhibit cross-resistance with related aldehyde and ketone derivatives of the dihydrotriazine series but not with 4-aminopteroylglutamic acid, 4-amino-N^{10}-methylpteroylglutamic acid or certain 2,4-diaminopyrimidines.

The results of variance analyses were compatible with the conclusion that dihydrotriazine resistance originates from spontaneous mutations independently of the inhibitor. However, the possibility that resistance to minimal concentrations of inhibitor may be an adaptive change could not be excluded.

---

1. Farber, S., *Blood,* 1952, v7, (Suppl.).

2. Law, L. W., and Boyle, P. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 599.

3. Burchenal, J. H., Robinson, E., Johnston, S. F., and Kushida, M. N., *Science,* 1950, v111, 116.

4. Law, L. W., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 340.

5. Burchenal, J. H., Webber, L. F., Meigs, G. M., and Biedler, J. L., *Blood,* 1951, v6, 337.

6. Burchenal, J. H., Johnston, S. F., and Waring, G. B., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 348.

7. Law, L. W., *Blood,* 1952, v7, (Suppl.), 160.

8. Burchenal, J. H., *Blood,* 1952, v7, (Suppl.), 166.

9. Kidder, G. W., Dewey, V. C., and Parks, R. E., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 88.

10. Foley, G. E., *Blood,* 1952, v7, (Suppl.), 165.

11. Burchenal, J. H., Waring, G. B., and Hutchison, D. J., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 311.

12. Foley, G. E., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 57.

13. ———, *ibid,* 1953, v83, 62.

14. Halvorsan, H. O., and Ziegler, N. R., *Quantitative Bacteriology,* ed. 1938, Burgess Pub. Co., Minneapolis, Minn.

15. Luria, S. E., and Delbrück, M., *Genetics,* 1943, v28, 491.

16. Hitchings, G. H., Rollo, I. M., Goodwin, L. G., Coatney, G. R., *et al., Trans. Royal Soc. Trop. Med. and Hyg.,* 1952, v46, 467.

17. Demerec, M., *Proc. Nat. Acad. Sci. U. S.,* 1945, v31, 16.

18. ———, *Ann. Missouri Botan. Garden,* 1945, v32, 131.

19. ———, *J. Bact.,* 1948, v56, 63.

20. Oakberg, E. F., and Luria, S. E., *Genetics,* 1947, v32, 249.

21. Witkin, E. M., *Genetics,* 1947, v32, 221.

22. Burnet, F. M., *J. Path. and Bact.,* 1929, v32, 15.

23. Lederberg, J., and Lederberg, E. M., *J. Bact.,* 1952, v63, 399.

24. Eagle, H., Fleischman, R., and Levy, M., *J. Bact.,* 1952, v63, 623.

25. Sevag, M. G., and Rosanoff, E. I., *J. Bact.,* 1952, v63, 243.

26. Gibson, M. I., and Gibson, F., *Nature,* 1951, v167, 113.

27. Schmith, K., *Acta Path. et Microbiol., Scand.,* 1943, v20, 563.

28. Saz, A. K., Eagle, H., and Toal, J. N., *J. Bact.,* 1952, v63, 513.

29. Julius, H. W., *Ann. Rev. Microbiol.,* 1952, v6, 411.

Received July 9, 1953. P.S.E.B.M., 1953, v83.

Effect of Food and Water on Thyroid Uptake of Radioiodine (I^{131}) in Rats. (20480)

JOHN P. STORAASLI, SAUL ROSENBERG, AND HYMER L. FRIEDELL.
From the Department of Radiology, Western Reserve University, Cleveland, O.*

Based on the avidity of the thyroid gland for iodine, diagnostic tests using tracers of radioactive iodine have been devised for studying patients with thyroid disease. Many variations of these tests have been described in the last few years, ranging from measurements of early accumulation rate over the thyroid gland to 24-hour uptake studies(1-4). The accumulation rate of iodine in the thyroid gland is in part dependent upon the plasma level of iodine. Therefore, alterations in plasma concentration may produce changes in the accumulation rate of iodine in the thyroid. Since plasma levels might be affected by variation in gastrointestinal or renal activity, and since no standard procedures having to do with restricted amounts of food or fluid are instituted during routine thyroid studies, it seems advisable to study the effects of these substances on the total thyroid accumulation of a tracer dose of radioactive iodine.

Method. Male albino rats, C. F. Wistar strain, were used. The animals weighed from 120 to 150 g at the onset of the experiment. For a period of 4 weeks prior to the study, the animals were placed on distilled water and an iodine-poor diet. The diet was similar to that used by Levine and Remington(5) in their studies on goiter production in rats.[†] It was found necessary to use an iodine-poor diet for this experiment since stock diet contains sufficient stable iodine to completely saturate the rat thyroid. The animals were separated into 8 groups of 3 animals, and were given single intraperitoneal injections of 0.1 μc of radioactive iodine per g of body weight. All animals were placed in metabolism cages and their excreta collected throughout the entire period of the experiment. Groups 1-A and 1-B received no water or diet food for 36 hours prior to the administration of the I^{131} and none during the period of the experiment. Groups 2-A and 2-B received diet food and water up to the time of injection but received none after the injection of I^{131}. Groups 3-A and 3-B received diet food and water up to the time of injection, but only distilled water after the injection. Groups 4-A and 4-B received diet food and water *ad lib.* prior to and following the injection of I^{131}. Animals in A groups were sacrificed at the end of 6 hours, and those in B groups at the end of 24 hours. The tissues to be studied were removed and dissolved in 30% potassium hydroxide. The excreta and carcasses were similarly treated. The radioactivity of aliquots of the various tissues were compared with standards. Measurements of gamma ray activity were made

---

* Most of the work performed under Atomic Energy Commission Contract W-31-109-eng-78 with Western Reserve University.

[†] This diet is the Rachitogenic Diet II (with added irradiated yeast) supplied by the Nutritional Biochemical Co. It contains ground gluten 20%, ground whole yellow maize 76%, calcium carbonate 3%, and sodium chloride 1%.

TABLE I. The Effect of Food and Water on the Distribution of Radioactive Iodine.

	6 hr				24 hr			
Group No.	1A	2A	3A	4A	1B	2B	3B	4B
Thyroid	56.1	51.3	30.2	18.1	40.4	36.1	15.8	7.0
Excreted	5.6	10.2	22.8	36.0	42.9	45.5	60.0	68.9
Stomach & contents	1.1	0.6	4.1	7.8	0.5	0.5	1.3	1.6
Remaining viscera	8.8	7.8	12.9	21.2	2.6	2.4	3.5	5.7
Carcass	16.0	17.1	15.8	14.6	10.5	12.1	9.6	10.2
Total recovery	87.6	87.0	85.8	97.7	96.9	96.6	90.2	93.4

Group No. 1, A & B. No food and water for 36 hr prior to and after inj.
2, No food and water after inj.
3, Distilled water only, after inj.
4, Diet food and water, *ad lib.*

with a scintillation counter and the results recorded in per cent of administered dose per total organ.

Results and discussion. As was previously shown by Levine and Remington(5), the low-iodine diet utilized in this experiment produced some enlargement of the thyroid gland in the test animals. The average thyroid weight of these animals was 170 mg/kg body weight as compared to control animals on stock diet whose thyroid weights were found to be an average of 120 mg/kg body weight. Histologically, there was evidence of moderate epithelial hyperplasia of the glandular elements with slight vacuolation of the colloid within the follicles.

Table I gives the 6- and 24-hour distributions of radioactive iodine in the 8 groups of animals, the results being averaged for each group. Fig. 1 graphically illustrates the effect of water alone, and food and water, on the thyroid uptake and excretion of radioactive iodine at 6 and 24 hours. It is obvious from the data that water alone, and food and water, have a profound effect upon the accumulation of I^{131} in the thyroid gland. In Fig. 1 it can be seen that at the end of 6 hours the uptake of I^{131} in the thyroid gland is 56 and 51% respectively in those groups (1-A and 2-A) where no food or water were given following the administration of the tracer dose. On the other hand, 30% of the tracer dose was observed to be in the thyroid tissue in those animals receiving only fluid (3-A); and 18% in animals given both food and water (4-A). Similar findings were observed at the end of 24 hours with a more profound effect due to food and water.

To obviate the conclusion that food and water given to the animals in Groups 3 and 4 contained sufficient stable iodine to influence and compete with the tracer dose of I^{131}, samples of diet food and distilled water were analyzed for iodine content by the method of Barker *et al.*(6). The diet food was found to contain an average of $14.6 \pm 1.7\ \gamma$ per kilo of food; and distilled water was found to contain 1.5 γ per liter. Estimating that a 250 g rat will consume approximately 20 to 30 g of food, and from 20 to 30 cc of water in a 24-hour period, the amount of exogenous iodine from the food and water would amount to approximately 0.3 γ. In order to be certain that this amount of stable iodine was not influencing the thyroid uptake of the tracer amount of radioactive iodine, 6 animals were divided into 2 groups, half receiving carrier-free I^{131} and the other half receiving 1.5 γ of stable iodine plus the tracer dose. After 24 hours the animals were sacrificed and analysis of the thyroid tissue and excreta for radio-

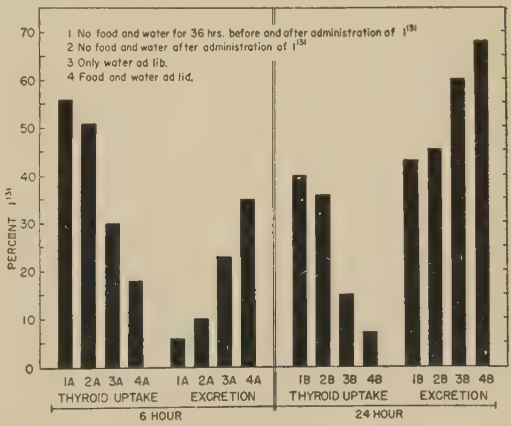


FIG. 1. Shows the influence of food and water alone on the thyroid retention and excretion of 1^{131}.

activity showed no significant difference.

The ability of water alone to produce a marked reduction in thyroid accumulation of I^{131} is intriguing. Although the exact mechanism is obscure, it must be assumed that renal excretion plays a great part. It is well known that following the administration of a tracer dose of radioactive iodine, the kidneys rapidly eliminate all iodine that is not retained in the thyroid. The results of this experiment indicate that one can markedly increase the total excretion of a tracer dose of radioactive iodine by simply allowing the animals to ingest fluid.

The mechanism by which food and water affect the distribution of I^{131} is again not well understood. Whereas fluid alone will produce a reduction in the final thyroid uptake (Groups 3-A and B), the addition of food further reduces the thyroid accumulation, and conversely increases the excretion of the tracer dose. It has been shown that this secretory activity of the stomach will alter or reduce the early accumulation rate of I^{131} in the thyroid gland(7). However, because of the rapid reabsorption of iodide ion by the gastrointestinal tract, it has been previously assumed that the ultimate retention of the tracer dose in the thyroid gland will not be affected. The fact that the per cent excreted in Group 4 is much greater than in Groups 1, 2, and 3 shows that gastric activity alone in some way reduces the final uptake of I^{131} in the rat thyroid gland.

It should be mentioned that the results obtained in this experiment may pertain only to the iodine-deficient rat and need not apply to a normal rat or one on a maintenance dose of iodine. However, from a preliminary study in rats fed a weight maintenance diet of stock food (Frisky pellets), it was found that food and water influenced the retention of I^{131} in the thyroid gland. In the control group (those receiving no food or water after the tracer dose was given), a 12% retention of I^{131} in the thyroid was obtained. In a second group (those given food and fluid after injection of the tracer dose), the retention of I^{131} was found to be only 6%.

Because of the results obtained in rats, it appears reasonable to assume that certain control measures should be instituted when thyroid activity is to be evaluated clinically. In studying early accumulation rates of I^{131} over the thyroid gland in patients, we have found it necessary to withhold food and fluid for several hours prior to the test and during the early measurement period. We have found that food in the stomach will definitely retard the early accumulation rate of I^{131} over the thyroid gland. These findings are considerably more evident in patients with over-active goiters, in whom the bulk of the tracer dose is rapidly accumulated in the thyroid gland. It has been much less evident, however, in euthyroid patients where not only the accumulation rate but also the total retention of I^{131} are considerably lower. Whether the final thyroid retention of the tracer dose is similarly affected as well, is still to be determined.

Summary. 1. Data have been presented which show that intake of food and water, and water alone, influence the uptake of radioactive iodine in the thyroid gland of rats on an iodine deficient diet of Remington and Levine. At 6 and 24 hours, the thyroid uptake is reduced significantly if water is given. An even more profound reduction in uptake and correspondingly increased excretion has been observed if both food and water are allowed. 2. It has been suggested that gastric activity and fluid intake should be controlled when radioactive iodine uptake is to be used for evaluating and treating thyroid disease.

We are indebted to Mr. Frank MacIntyre of Veterans Administration Hospital of Cleveland for making the determinations of the stable iodine content of diet food and distilled water used in this experiment.

---

1. Werner, S. C., Hamilton, H. B., Leifer, E., and Goodwin, L. D., *J. Clin. Endocrinol.*, 1950, v10, 1054.

2. Myant, N. B., Pochin, E. E., and Goldie, E. A. G., *Clin. Sc.*, 1949, v8, 109.

3. Pochin, E. E., *Lancet*, 1950, v2, 41.

4. Berson, S. A., Yalow, R. S., Sorrentino, J., and Roswit, B., *J. Clin. Invest.*, 1952, v31, 141.

5. Levine, H., Remington, R. E., and von Kolnitz, H., *J. Nutrition*, 1933, v6, 325.

6. Barker, S. B., Humphrey, M. J., and Soley, M. H., *J. Clin. Invest.*, 1951, v30, 55.

7. Storaasli, J. P., unpublished data.

Received December 4, 1952. P.S.E.B.M., 1953, v83.

Utilization of Iodine by the Thyroid of the Platyfish, *Xiphophorus* (*Platypoecilus*) *maculatus*. (20481)

Olga Berg and Aubrey Gorbman.

From the Departments of Zoology, Barnard College and Columbia University, New York City.

The first experimental demonstration of the relation of iodine to thyroid function was made with teleost fish(1,2) in 1910, but since then teleost thyroidal physiology has received relatively little attention. A revival of interest in the comparative physiology of thyroid tissue has been made possible by availability of radioiodine and has resulted in recent work showing that marine teleost and elasmobranch thyroids can accumulate isotopic iodine and synthesize radiothyroxine (3-11). In regard to the function of the thyroid gland of adult freshwater teleosts, two important questions remain unanswered. 1) To what degree can the thyroid tissue accumulate iodide? 2) In what way is such iodide metabolized in the synthesis of thyroxine? In the experiments reported here, the freshwater killifish *Xiphophorus (Platypoecilus) maculatus* was used and quantitative data were sought to demonstrate the thyroidal accumulation rate and turnover of iodine given as iodide. By chromatographic analysis of thyroidal hydrolysates representing different time intervals after I^{131} administration, the varying proportions of iodide and iodinated amino acids during thyroxine synthesis was studied.

Materials and methods. Adult sibling platyfish, *Xiphophorus maculatus,* all of the same sex and 25 to 35 mm in standard length, were carefully chosen from inbred laboratory stocks* to give maximum animal unifomity. They were kept at all times in water taken from the general laboratory supply in which this stock is maintained. Twenty μc of I^{131} was given to each fish intraperitoneally. Groups of 4 fish were sacrificed at definite post-injection intervals and thyroid tissue (the entire lower jaws) was pooled for analysis for each group. The variation of the procedure in each of 5 separate experiments is summarized in Table I.

Thyroidal iodine uptake and loss, over a period of time, was followed by measuring radioactivity of aliquots of alkali-hydrolysed tissue samples. Radioactivity of the water in which animals were kept also was determined, and all results expressed in terms of per cent of the original dose given.

Extraction of the thyroidal hydrolysates and their analysis by filter paper chromatography was done by standard technics, and closely followed the procedure of Roche *et al.* (12) and Lissitzky(14). Two different developing solvents were used, n-butanol-acetic acid, and n-butanol-ammonia. As known reference substances, the following were employed: monoiodotyrosine, diiodotyrosine, diiodothyronine, d-l thyroxine, and potassium iodide. These also were used, in some instances, as "carriers". For the observation of the distribution of the radioactivity along the length of the filter paper chromatograms, a Geiger tube and shielding arrangement, resembling that of Lissitzky(14), was used.

Radioautography of paraffin sections of thyroid tissue of I^{131} injected *X. maculatus* was done by exposing such sections to squares of x-ray film.

Results. In the 4 experiments with fishes in normal stock water, relatively rapid, though somewhat variable, thyroidal uptake of radioiodine was found (Table II). The maximum uptake, 29%, occurred 24 to 48 hours after injection. The observed variation seemed to bear no relation to whether the water was changed frequently or whether the fishes were allowed to remain in contact with their excreted radioiodine. Even after 144 hours, and despite frequent water changes, the thyroids retained about 75% of their peak quantities of I^{131}, indicating that turnover of metabolized I^{131} was quite slow.

Since thyroidal uptake was so relatively high, and release of accumulated iodine so

---

* All fish were generously supplied by Dr. Myron Gordon, Genetics Laboratory, New York Aquarium, New York Zoological Society.

TABLE I. Summary of Experimental Procedure.

Strain	No., sex of animals	Culture conditions	Treatment	Time of sacrifice after exposure to radioiodine in hr 2	6	12	24	48	96	144	Type of hydrolysis
405	24 ♀♀	Groups of 4 fish in 400 cc water. Sacrificed together	Water not changed	*	*	*	*	*	*		2 N NaOH 18 hr 100°C
308	24 ♀♀	As in exp. 1	As in exp. 1	*	*	*	*	*	*		10 mg trypsin 48 hr pH 8.5 37°C
394	24 ♀♀	" " " "	Water changed once every 6 hr for 24 and once every 12 hr thereafter	*	*	*	*	*		*	1 N NaOH 16 hr 100°C
407	16 ♀♀	Groups of 8 fish in 500 cc water. Sacrificed 4 at a time	Water changed 3 times before the 12 hr and 24 hr sacrifice and 5 times before the 48 hr and 144 hr sacrifice			*	*	*		*	8% Ba(OH)$_2$ 13 hr 100°C
375	12 ♂♂	As in exp. 1	Treatment with 24 mg of KI/400 cc water initiated 1 wk prior to inj., continued until sacrifice. Water containing KI changed twice prior to inj., thereafter as in 394.			*	*		*		None

TABLE II. Uptake of Radioactive Iodine by Fish Thyroid Tissue, Expressed as % of Total Dose Injected.

	Water not changed		Water changed*		60 p.p.m. KI solution changed*
Strain	405	308	394	407	375
Time, hr					
2	5.8	13.1	6.5		
6	10.8	17.4	10.1		
12	11.3	19.6	19.6	15.0	2.9
24	14.1	24.4	16.4	29.5	3.1
48	14.7	29.9	16.8	19.5	
96	18.0	17.3			1.6
144			16.5	16.4	

* For details of intervals between changes of water, see Table I.

slow, it seemed possible that the fish were "iodine-starved". To test this an additional series of observations was made on animals kept in water which was 60 parts per million KI(4). They were placed in this solution one week prior to I^{131} injection and kept in it continuously until sacrificed. With this treatment the maximum thyroidal I^{131} uptake was only 3% of the injected dose. However, loss of this relatively small amount of radioiodine from the thyroid was slow as in the previous experiment.

As a correlate of the data on thyroidal iodine accumulation and loss, Fig. 1 shows the loss of I^{131} into the water by members of *X. maculatus* strains 407 and 375. Presumably, the differences between these sets of data and the data on thyroidal I^{131} indicate the amounts of radioiodine in the extra-thyroidal tissues of the animals. It may be

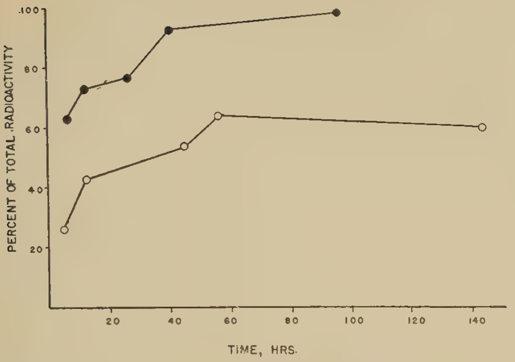


FIG. 1. The loss of radioactive iodine into water from fish injected with tracer dose of I^{131}. White circles: fish kept in small amount of water changed frequently (Table I). Black circles: fish treated with potassium iodide for one week before being given I^{131}. Water also changed frequently.

noted that the greatest rate of loss of I^{131} into the aquarium water from the injected fishes was during the first 12 hours. Whereas fish in iodide-enriched water had lost almost all of the injected I^{131} by 2 days, fish in normal (iodide-poor?) water still retained about 40% of the injected I^{131} in their thyroids and body tissues as long as 6 days after injection.

Radiochromatography. Different authors have recommended different hydrolytic technics for dissociation of the protein bound iodinated amino acids in thyroid(12-14). Because it is yet uncertain which method is most advantageous, 3 were used. As expected from the literature(13,14) NaOH-hydrolysis gave the highest iodide and lowest thyroxine values, presumably because it deiodinates thyroxine fairly readily. Trypsin gave relatively the lowest iodide and higher thyroxine figures, but it is not certain that in our hands it achieved complete hydrolysis. Barium hydroxide (8%) gave intermediate results. Despite these technical variables a unified picture can be constructed from the available data (Fig. 2) showing that thyroxine synthesis follows the same pattern in *Xiphophorus* as in mammals. Although the increase in radioiodide was not striking, radiothyroxine tended to increase, but on a small scale. Monoiodotyrosine and diiodotyrosine shifted in relative importance with time, MIT predominating earlier; DIT later. It is interesting that the largest proportion of radiothyroxine seen in any of the experiments was 15%.

Histology and radioautography. All the fish examined histologically had normal thyroid tissue. The follicles varied greatly in size. The epithelial cells were usually low cuboidal but in the largest follicles they were squamous. In most cases the colloid was dense, eosinophilic, and granular, but in some of the larger follicles it appeared to be nongranular. Especially strong radioautographs were made by the colloid. Unfortunately, with the autographic technic used it was difficult to determine whether the epithelium contained appreciable quantities of radioiodine.

Discussion. The four lower curves in Fig. 3 represent marine fishes; the 4 upper curves, freshwater species; the intermediate curve 5, the seaward migratory "smolt" form of the salmon. If such data, drawn from the work of several groups of investigators, can be taken to differentiate a freshwater from marine type of thyroid, it is possible that this variation is due to the differences in environmental iodine of such fishes. Both LaRoche (4) with immature salmon, and we, with adult platyfish, have demonstrated a decreased I^{131} uptake in freshwater fishes kept in iodine-enriched water. Such experimental curves, if plotted on Fig. 3, would fall within the family of curves representing the marine fish group. It may be noted that the Roussette shark(8), though it briefly accumulates a relatively high percentage of administered I^{131}, loses it promptly. A similar rapid loss of I^{131} was seen in our experiment with fish in iodine-enriched water (Fig. 1).

The data summarized in Fig. 2 show that synthesis of thyroxine in *Xiphophorus* follows the same pattern as it does in mammals(14), or in the shark(8). Monoiodotyrosine and diiodotyrosine are the precursors for thyroxine, with monoiodotyrosine being in excess in earlier phases and diiodotyrosine in excess in later intervals. It seems remarkable that despite the relatively high degree of thyroidal I^{131} accumulation, only a small proportion of it (maximum, 15%) was in the form of thyroxine as late as 6 days after injection. This could mean either that thyroxine synthesis is

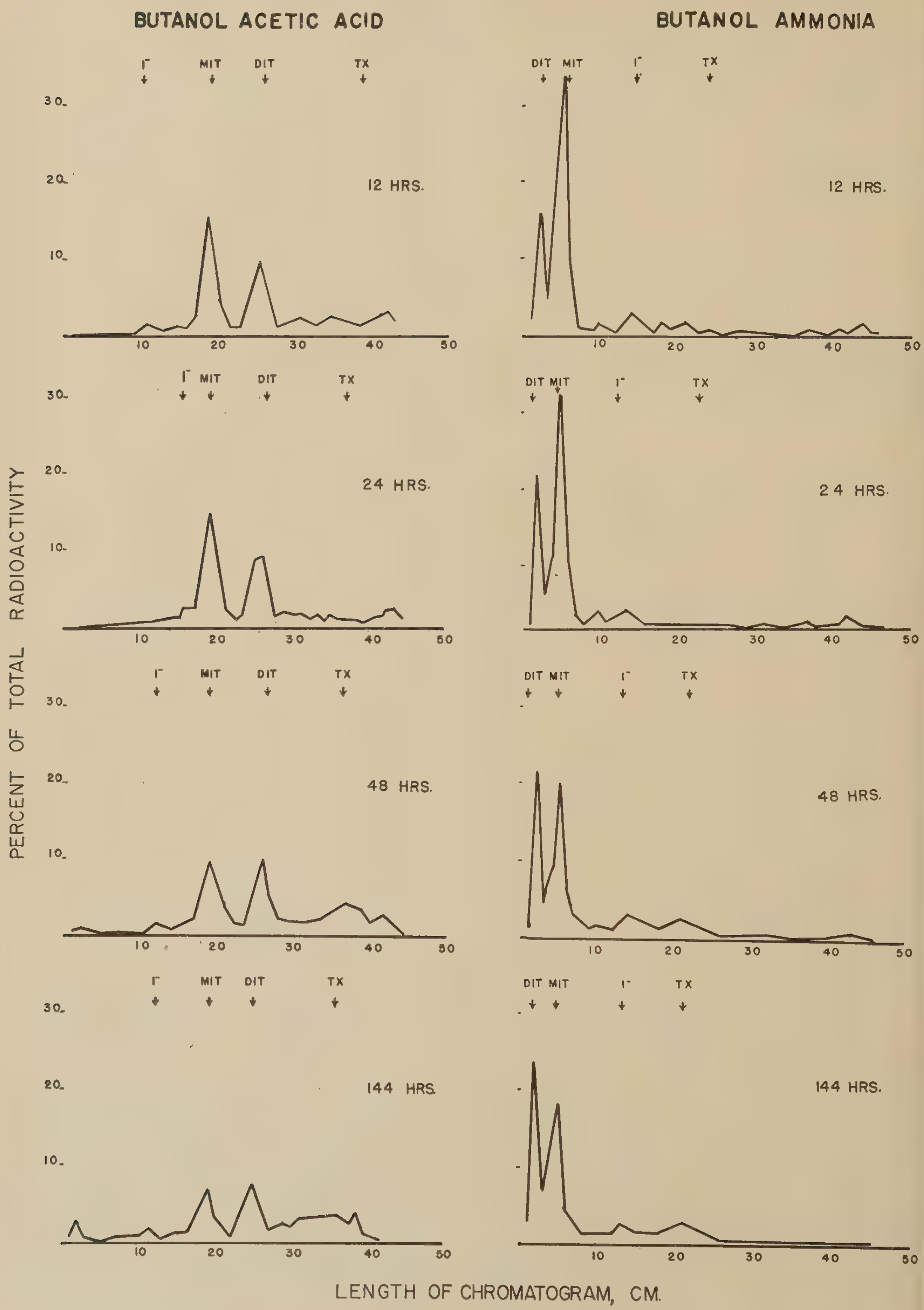

BUTANOL ACETIC ACID
BUTANOL AMMONIA
I⁻
MIT
DIT
TX
12 HRS.
24 HRS.
48 HRS.
144 HRS.
30
20
10
10
20
30
40
50
PERCENT OF TOTAL RADIOACTIVITY
LENGTH OF CHROMATOGRAM, CM.

FIG. 2. Radiochromatograms of barium hydroxide hydrolysates of thyroid tissue of platyfish sacrificed at intervals after injection of a tracer dose of radioactive iodine. Figures show distribution of radioactivity along length of chromatograms. Probable chemical form of I^{131} is indicated at top of each figure. Arrows indicate position of known iodinated compounds chromatogrammed as carriers or in parallel with thyroid hydrolysates. I, iodide; MIT, monoiodotyrosine; DIT, diiodotyrosine; Tx, thyroxine.

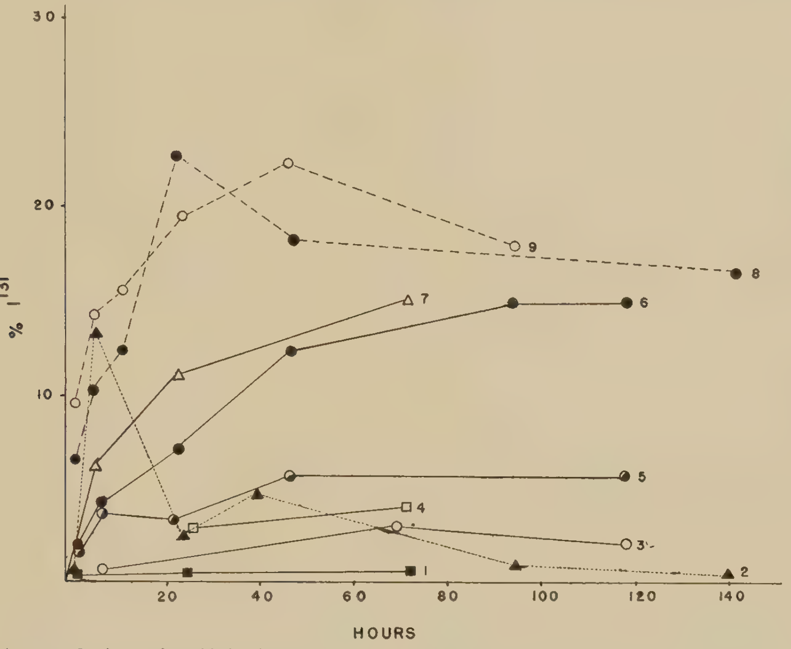


FIG. 3. Accumulation of radioiodine by thyroid glands of various fishes. Curves 3 and 4 are for marine teleosts whose thyroidal uptake of I^{131} is low or the turnover is rapid. Curves 1 and 2 for marine lamprey and shark, respectively, are similar. Curve 5 represents data from salmon smolt, caught in fresh water just before downstream migration. Note that I^{131} uptake resembles that of a marine fish. Curves 6 and 9 are for fresh water teleosts; I^{131} uptake is high and its loss from the thyroid is slow. 1. Marine lamprey, *Petromyzon marinus*, redrawn from Leloup(6). 2. Roussette shark, *Scyliorhinus canicula*, drawn from data of Gorbman *et al.*(8). 3. Marine mullet, *Mugil auratus*, drawn from data of Leloup(7). 4. Marine conger eel, *Conger conger*, drawn from data of Leloup(7). 5. Salmon smolt, *Salmo salar*, drawn from data of Fontaine *et al.*(5). 6. Salmon parr, *Salmo salar*, drawn from data of Fontaine *et al.*(5). 7. Salmon parr, redrawn from LaRoche(4). 8. Platyfish, *Xiphophorus maculatus*, water changed frequently during experiment. 9. Platyfish, *Xiphophorus maculatus*, kept in same water for duration of experiment.

very slow, or that thyroxine is selectively released from the thyroid as it is formed. Our data do not favor either alternative explanation.

Summary. 1. One hundred platyfish, *Xiphophorus (Platypoecilus) maculatus*, 12 pretreated with potassium iodide, were injected with a tracer dose of carrier-free I^{131} and sacrificed at intervals up to 144 hours after injection. Groups of 4 fish were processed together. The lower jaws, containing the thyroidal tissue, were removed, hydrolysed, and measured for I^{131} uptake. Such hydrolysates then were analyzed by chromatographic techniques and the relative rates of synthesis of monoiodotyrosine, diiodotyrosine, and thyroxine were determined. 2. The thyroid tissue accumulated about one-third of the injected isotope. Uptake was rapid but loss was slow so that 144 hours after injection 15% of the I^{131} was still held in the thyroid. Thyroid tissue of fish kept in 60 p.p.m. KI solution accumulated only 3% of the injected dose of I^{131}. 3. The loss of radioiodine into the water was much more rapid in fish treated with KI than in the others. 4. As in mammals, the thyroid first synthesized monoiodotyrosine, then diiodotyrosine, and finally, thyroxine.

The synthesis of thyroxine was relatively slow. A maximum value of 15% of the radioiodine in the thyroid was converted to thyroxine under the experimental conditions employed.

1. Marine, D., and Lenhart, C. H., *J. Exp. Med.*, 1910, v12, 311.

2. ———, *J. Exp. Med.*, 1911, v13, 455.

3. Matthews, S. A., and Smith, D. C., *Am. J. Physiol.*, 1948, v153, 222.

4. LaRoche, G., *Ann. de l'ACFAS,* 1950, v16, 134.

5. Fontaine, M., Leloup, J., and Olivereau, M., *Arch. des Sciences Physiol.*, 1952, v6, 83.

6. Leloup, J., *J. de Physiol.*, 1952, v44, 284.

7. ———, *Compt. rend. Soc. Biol.*, 1952, v146, 1014.

8. Gorbman, A., Lissitzky, S., Michel, R., and Roche, J., *Endocrinology,* 1952, v51, No. 4, 311.

9. Vivien, J. M., and Gaiser, M. L., *Comp. rend. Acad. Sci.*, 1952, v234, 1585.

10. Wolff, J., and Chaikoff, I. L., *Endocrinology,* 1947, v41, 295.

11. Leloup, J., *Comp. rend. Acad. Sci.*, 1951, v233, 635.

12. Roche, J., Justisz, M., Lissitzky, S., and Michel, R., *Compt. rend. Soc. Biol.*, 1950, v144, 1321.

13. Michel, R., Thesis, Lons-le-sauner, Imprimerie M. Declume, 1950.

14. Lissitzky, S., Thesis, Paris, A. I. A., 1952.

Received April 21, 1953. P.S.E.B.M., 1953, v83.

Effect of Dihydrocholesterol (Cholestanol) Administration on Plasma Cholesterol and Atherosclerosis in the Rabbit.* (20482)

C. W. NICHOLS, JR., M. D. SIPERSTEIN, AND I. L. CHAIKOFF.

From the Department of Physiology of the University of California School of Medicine, Berkeley.

We reported recently that the administration of dihydrocholesterol (DHC) to chickens fed a cholesterol-containing diet prevented the development of both hypercholesterolemia and the associated atherosclerosis(1). Since the feeding of this saturated steroid might offer a means of inhibiting the atherogenic effects of dietary cholesterol, it seemed desirable to extend our observations to mammals. The present report deals with the action of DHC in the rabbit.

Experimental. Rabbits weighing from 1850 to 2270 g were fed an adequate commercial pellet diet† for 6 weeks before being placed on the experimental diets recorded in Table I. During these 6 weeks, 2 blood samples were obtained from each animal by cardiac puncture, and the total cholesterol content of plasma was determined by the method of Sackett(2). At the end of this period, the rabbits were divided into 4 groups, and fed as shown in Table I. These experimental diets were fed *ad lib.* for a period of 2 weeks at the end of which time—because the supply of DHC was exhausted—all rabbits were placed on the normal pellet diet for the next 5 weeks. At week 13 the level of plasma cholesterol was again determined, and the feeding of the experimental diets resumed (Table I). The experiment was continued for 9 more weeks, and during this time 4 additional plasma cholesterol values were obtained. Each group consumed approximately the same amount of diet.

TABLE I. Composition of Diets. All diets consisted of commercial rabbit pellets to which was added one or more of the following constituents:

Constituent	Group I	II	III	IV
	%			
Wesson oil	5	5	5	5
Cholesterol	0	1	1	0
DHC*	0	0	2	2

* The dihydrocholesterol used in this study was generously supplied by the Schering Corp.

---

* Aided by grants from the Life Insurance Medical Research Fund and the U. S. Public Health Service.

† This diet contained 60% alfalfa meal, 10% ground barley, 10% cottonseed meal, 10% rice polishings, 4% watergrass seed, 4% rice seed, 1% oyster shell flour, 0.5% cane molasses, and 0.5% salt.

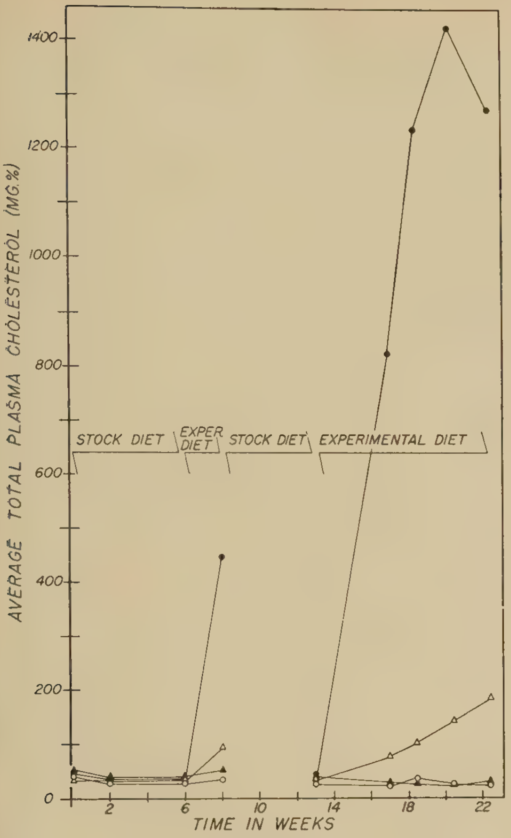


FIG. 1. Influence of DHC feeding upon plasma cholesterol levels of the normal and the cholesterol-fed rabbit. ○—Group I. Average plasma cholesterol levels observed in 8 rabbits fed a basal diet (commercial rabbit pellets plus 5% Wesson oil). ●—Group II. Average plasma cholesterol levels found in 8 rabbits fed a basal diet plus 1% cholesterol. △—Group III. Average plasma cholesterol levels in 7 rabbits fed a basal diet plus 1% cholesterol plus 2% DHC. ▲—Group IV. Average plasma cholesterol levels of 8 rabbits fed a basal diet plus 2% DHC.

The values for total plasma cholesterol are shown in Fig. 1. Although the administration of DHC to rabbits fed cholesterol (Group III) did not completely prevent a rise in plasma cholesterol, it is nonetheless clear that these rabbits exhibited a much less pronounced rise in total plasma cholesterol than did those fed cholesterol alone (Group II). It should be noted that the addition of DHC to a *normal* diet did not depress the level of plasma cholesterol below those found in normal animals. Thus the average values for plasma cholesterol were 28 mg % in the DHC-fed animals and 31 mg % in control animals. DHC apparently acts upon the hypercholesterolemia of exogenous origin for it seems to have no effect upon the cholesterol level of the normal rabbit.

The rabbits were sacrificed at the end of the experiment, and their aortas and livers were removed. Each atherosclerotic plaque was carefully measured, and the total area estimated. These findings are summarized in Table II. Most of the rabbits fed the 1% cholesterol diet developed atherosclerosis. The average area of the atherosclerotic plaques found in this group (II) was 271 mm². The addition of DHC to the 1% cholesterol diet reduced this figure to 5.1 mm². Two of the 8 rabbits (Group IV) that were fed DHC with a normal diet developed atherosclerotic plaques. No atherosclerosis was observed in the untreated rabbits.

Total lipids for each liver sample were determined as follows: Liver samples were first hydrolyzed by treatment with a saturated sodium hydroxide-ethanol mixture on a steam bath for 8 hours. The mixture was then acidified and extracted 3 times, each time with a 10-fold excess of petroleum ether. The pooled petroleum ether extracts were transferred to tared flasks and evaporated to dryness on a steam bath. The weight of the residue was then determined. The values for total liver lipids of the untreated rabbits (Group I) averaged 3.0% (2.6-3.3) while those for the DHC-treated rabbits (Group IV) averaged 3.3% (2.1-4.6). The average values for liver lipids were 6.7% (3.2-9.3) for the rabbits fed cholesterol (Group II) and 4.6% (1.1-7.9) for those fed cholesterol plus DHC (Group III).

Comment. It is clear that the enteral administration of DHC is effective in inhibiting experimentally-induced atherosclerosis in the rabbit as well as in the bird. Under the conditions of our study, the DHC did not prevent completely the hypercholesterolemia of cholesterol feeding nor did it prevent a small increase of lipids in the livers of rabbits fed *both sterols*. It should also be noted that the formation of atherosclerotic plaques in rabbits fed DHC alone (Table II) is not ruled out. This phase is under further study.

TABLE II. Effect of Dihydrocholesterol upon Degree of Atherosclerosis in the Rabbit.

No. of rabbits	Diet	Gross atherosclerosis (mm²)	
8	No cholesterol (Group I)	0	
8	1% cholesterol (Group II)	140	
		18	
		0	
		16	Avg 271 mm²
		487	
		318	
		1176	
		10	
7	1% cholesterol + 2% dihydrocholesterol (Group III)	25	
		0	
		0	Avg 4.9 mm²
		0	
		9	
		0	
		0	
8	2% dihydrocholesterol (Group IV)	8	
		0	
		0	
		0	Avg 19.8 mm²
		150	
		0	
		0	
		0	

In the bird, an increase in the lipid content of the liver was observed after prolonged feeding of a diet containing 2% DHC.[‡] In the rabbit, however, the feeding of a 2% DHC diet for as long as 2 months failed to affect the lipid content of the liver. The tissues of rats fed a diet containing 10% DHC for 8 weeks were examined histologically by Dr. Stuart Lindsay of the Pathology Department; no abnormalities were observed.

Summary. 1. Effects of dihydrocholesterol on cholesterol content of plasma and on development of atherosclerosis were investigated in the rabbit. 2. Rabbits fed a diet containing 1% cholesterol exhibited typical hypercholesterolemia (average, 1041 mg/100 cc of plasma) and atherosclerosis (average estimated plaque area was 271 mm²). Rabbits fed 1% cholesterol plus 2% dihydrocholesterol, the average plasma cholesterol value was 120 mg % and the average atherosclerotic plaque area was 4.9 mm². 3. Addition of dihydrocholesterol to a normal diet had no effect on the levels of plasma cholesterol, but did result in an increase in the average atherosclerotic plaque area.

The technical assistance of J. Sherokoff is gratefully acknowledged.

[‡] The lipid content of the livers of birds fed a diet containing 2% DHC for 12 weeks ranged from 6.5 to 9.7 (average, 7.8%). The range for control birds was 2.9 to 5.7 (average 4.0). (Unpublished observations.)

1. Siperstein, M. D., Nichols, C. W., Jr., and Chaikoff, I. L., *Circulation*, 1953, v7, 37.

2. Sackett, G. E., *J. Biol. Chem.*, 1925, v64, 203.

Received May 26, 1953. P.S.E.B.M., 1953, v83.

Anterior Pituitary Growth Hormone (STH) and Pancreatic Secretion of Glucagon (HGF).[*] (20483)

Piero P. Foa, Edward B. Magid, Morton D. Glassman, and Harriet R. Weinstein.

From the Department of Physiology and Pharmacology, The Chicago Medical School.[†]

Houssay and Biasotti(1) were the first to demonstrate that the removal of the pituitary gland ameliorates pancreatic diabetes. This discovery was followed by the observation that injections of crude anterior pituitary extracts(2), or purified growth hormone (somatotrophic hormone or STH(3)) cause temporary hyperglycemia and glycosuria, followed by permanent "metahypophyseal" diabetes. It is believed(4,5) that anterior pituitary hormone(s) first increase the demand for insulin and overstimulate the Islets of Langerhans and, if the injections are continued, cause a progressive degranulation, exhaustion, and degeneration of the beta cells, and a decreased secretion of insulin. In contrast to the beta cells, the alpha cells of animals with metahypophyseal diabetes appear to be normal (4-6). On the basis of evidence recently

---

[*] Supported by a grant from Eli Lilly and Co.

[†] 710 South Wolcott Avenue, Chicago.

reviewed(7,8), the alpha cells are believed to be the site of formation of the hyperglycemic-glycogenolytic factor of the pancreas (HGF or glucagon), probably a second pancreatic hormone secreted in response to hypoglycemia (9-12). For these reasons, two possible explanations for metahypophyseal diabetes are suggested: 1) decreased insulin production leaving the anti-insulin hormones (*e.g.*, glucagon) unopposed, and 2) stimulation of the alpha cells by anterior pituitary hormones with increased glucagon secretion. The experiments described in this paper were designed to study the second hypothesis.

Materials and methods. The problem was studied by means of 20 cross-circulation experiments. Mongrel dogs of both sexes were used. All animals were healthy, well fed, and received the last *ad libitum* meal about 16 hours preceding the experiment. The dogs were anesthetized with sodium pentobarbital (35 mg/kg), heparinized, and prepared as described previously(12). Due to the heparinization, the importance of painstaking surgical hemostasis cannot be overemphasized.

In 14 experiments, the pancreato-duodenal vein of a donor *dog D* was connected with a femoral vein of a recipient *dog R* by means of glass cannulae and polyethylene tubing. These 14 experiments were of 3 types: in 5 experiments, the donor dogs received an intravenous injection of STH at the time of the experiment; in 6 experiments, the donor dogs received a similar injection at the time of the experiment, but in addition, were pretreated with 6 daily intramuscular injections of STH; in 3 experiments, the donor dogs received a control intravenous injection of 10 cc of a dilute NaOH solution at the same pH (10) of the STH solution. In 6 additional control experiments, a mesenteric vein of *dog D* was anastomosed to a femoral vein of *dog R*, and *dog D* received an intravenous injection of STH at the time of the experiment.

Highly purified ACTH-free beef anterior pituitary STH (Armour batch No. R-285-183) was dissolved in 10 cc of dilute NaOH (pH 10). All intravenous and intramuscular doses were of 3,5 mg/kg. This dose caused a rapid and transient hyperglycemia in a few preliminary experiments in dogs and was of the same order of magnitude as that dose having a similar effect in normal men(13) and in mice with hereditary obesity(14). Blood glucose was determined according to Nelson's photometric adaptation of Somogyi's method(15).

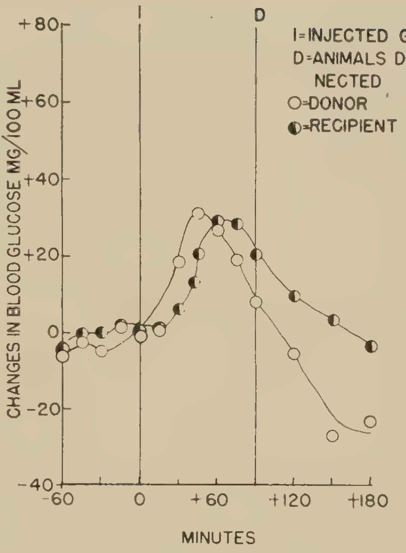


FIG. 1. Pancreatic-femoral anastomosis: Avg of 11 exp.

Results. The results are presented as curves showing average changes in blood sugar concentration. Fig. 1 represents the results obtained in 11 pancreatic-femoral cross-circulation experiments in which the donor *dog D* received an injection of STH at time 0. Of these 11 donor dogs, 5 had received no treatment previous to the experiment and 6 had received 6 daily injections of STH as mentioned above. Pre-treatment with STH did not modify the results significantly; the data, therefore, were averaged. During the control period preceding the injection, there were no significant changes in the blood sugar of either the donor *dog D* or the recipient *dog R*. Following the injection of STH into *dog D*, the blood sugar of both dogs began to rise, reaching significant hyperglycemic levels in 30 to 90 minutes and returning to the initial values in about 120 minutes. The average maximum rise was approximately 30 mg/100 cc of blood in both donor and recipient, corresponding to a 32% and 36%

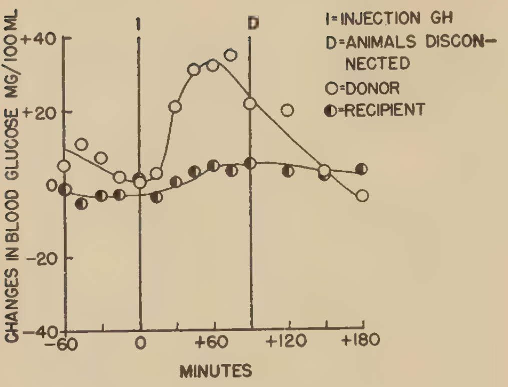


FIG. 2. Mesenteric-femoral anastomosis: Avg of 6 exp.

increase over the average blood sugar concentration at 0 time, respectively. This hyperglycemia was followed by hypoglycemia in 8 of the 11 donors and in 6 of the 11 recipients.

Fig. 2 represents 6 cross-circulation experiments in which a mesenteric vein of *dog D* and a femoral vein of *dog R* were connected. During the control period the blood sugar of both *dog D* and *dog R* remained essentially constant. Following the injections of STH into *dog D,* its blood sugar increased, reaching hyperglycemic levels in 60 to 90 minutes. The average maximum rise was again about 35 mg/100 cc of blood, or a 28% increase over the average blood sugar concentration at 0 time. The blood sugar returned to its original level in 120 to 150 minutes. This was followed by a hypoglycemia in 3 of the 6 donor dogs averaging 22% below the control level. This hypoglycemia occurred 120 to 180 minutes after the injection of STH and does not appear in the average curve because it occurred in only 3 out of 6 dogs, and not always at the same time. The blood sugar of the recipient dogs did not change significantly.

In 3 pancreatic-femoral cross-circulation experiments in which *dog D* received an injection of 10 cc of solution of NaOH at pH 10, instead of the solution of growth hormone, no significant changes in the blood sugar of either *dog D* or *dog R* were noted.

Discussion. The hyperglycemia observed in the recipient dogs following the injection of STH into the donors could be due to one or more of the following causes: 1) nonspecific causes, such as anesthesia or the general experimental procedure. This possibility is ruled out by the fact that the blood sugar remained reasonably constant during the preinjection control period and by the fact that when the donor dogs received alkaline water instead of STH, there was no rise in blood sugar in either *dog D* or *dog R;* 2) transfer of hyperglycemic blood from *dog D* to *dog R* through the anastomosis. This possibility is ruled out by the fact that the mesenteric blood of *dog D,* although containing the same amount of glucose as its pancreatic blood, did not cause hyperglycemia in *dog R;* 3) transfer of a significant fraction of the injected STH from *dog D* to *dog R.* This possibility similarly is ruled out by the fact that the mesenteric blood of *dog D* causes no hyperglycemia in *dog R;* 4) stimulation of the pancreas of *dog D* by the injected STH with secretion of a hyperglycemic factor (glucagon) and transfer of the material to *dog R* through the pancreatic-femoral anastomosis. This hypothesis, supported by the experimental evidence presented in this paper, is strengthened by the fact that the intensity and duration of the hyperglycemic response of *dog R* is similar to the intensity and duration of the response obtained in normal, non-anesthetized animals receiving intravenous injections of glucagon(10,11).

An increased secretion of glucagon can also explain the hyperglycemia observed in *dog D.* Although this dog loses part of its pancreatic blood, enough glucagon-containing blood, such as the blood coming from the head of the pancreas or from the gastric mucosa, does not leave the animal. Thus part of the glucagon produced by *dog D* remains in *dog D,* and part passes to *dog R,* causing hyperglycemia in both animals. The experimental data do not allow even an approximate estimate of the relative quantities of glucagon available to the 2 animals, since the intensity of the hyperglycemic response depends not only upon the amount of glucagon injected, but also upon the amount of liver glycogen present(16). Glucagon hyperglycemia has been shown to stimulate insulin secretion(12), and this is probably the cause of the hypoglycemia noted at the end of the experiments described here (Fig. 1).

We believe these results confirm and extend the findings of Bornstein, Reid, and Young(17), and give further support to the hypothesis that glucagon is a pancreatic anti-insulin hormone secreted not only in response to hypoglycemia, but also to the administration of anterior pituitary STH.

Summary and conclusions. Fourteen cross-circulation experiments were performed by anastomosing the pancreato-duodenal or a mesenteric vein of a donor *dog D* with a femoral vein of a recipient *dog R*. The intravenous injection of purified anterior pituitary STH into a normal donor or into a donor previously treated with the same STH causes the appearance of a hyperglycemic material (glucagon) in the blood of the pancreatic but not of the mesenteric vein. This glucagon causes hyperglycemia in a normal recipient dog. In addition to decreasing insulin secretion(5) and inhibiting peripheral utilization of glucose (18), STH appears to cause hyperglycemia by stimulating the secretion of glucagon by the pancreas. It is believed that these results further support the hypothesis that glucagon is a pancreatic anti-insulin hormone contributing to the maintenance of a normal blood sugar concentration.

We are indebted to Doctors Stanley Hier of the Wilson Laboratories for supplying Heparin, and to Doctors N. Ercoli, I. Bunding, and S. L. Steelman of the Armour Laboratories for supplying Heparin and STH.

---

1. Houssay, B. A., and Biasotti, A., *Rev. Soc. Arg. Biol.*, 1930, v6, 8; *Compt. Rend Soc. Biol.*, 1930, v104, 407; v105, 121, 124.

2. Young, F. G., *Acta Med. Scand.*, 1949, v135, 275.

3. Bennett, L. L., and Evans, H. M., in G. Pincus and K. V. Thimann, *The Hormones*, Academic Press, Inc., New York, 1950.

4. Houssay, B. A., and Foglia, V. G., *Rev. Soc. Arg. Biol.*, 1944, v20, 247; 1938, v4, 859.

5. Anderson, E., and Long, J. A., *Endocrinology*, 1947, v40, 98.

6. Dohan, F. C., Fish, C. A., and Lukens, F. D. W., *Endocrinology*, 1941, v28, 341.

7. Foà, P. P., *Chicago Med. School Quart.*, 1951, v13, 1.

8. Pincus, I. J., *Trans. N. Y. Acad. Sci.*, 1952, v14, 319.

9. Foà, P. P., Weinstein, H. R., and Smith, J. A., *Am. J. Physiol.*, 1949, v157, 197.

10. Foà, P. P., Berger, S., Santamaria, L., Smith, J. A., and Weinstein, H. R., *Science*, 1953, v117, 82.

11. Foà, P. P., Santamaria, L., Berger, S., Smith, J. A., and Weinstein, H. R., Proc. Soc. Exp. Biol. and Med., 1952, v80, 635.

12. Foà, P. P., Santamaria, L., Weinstein, H. R., Berger, S., and Smith, J. A., *Am. J. Physiol.*, 1952, v171, 32.

13. Carballeira, A., Elrick, H., MacKenzie, K. R., and Browne, J. S. L., Proc. Soc. Exp. Biol. and Med., 1952, v81, 15.

14. Meyer, J., and Silides, D. N., *Endocrinology*, 1953, v52, 54.

15. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

16. Burger, M., and Kramer, H., *Z. Ges. Exp. Med.*, 1929, v67, 441.

17. Bornstein, J., Reid, E., and Young, F. G., *Nature*, 1951, v168, 903.

18. Milman, A. E., DeMoor, P., and Lukens, F. D. W., *Am. J. Physiol.*, 1951, v166, 354.

Received June 8, 1953. P.S.E.B.M., 1953, v83.

Selective Sterilization of Young Female Mice by Radioiodine Transmitted Through the Mother's Milk. (20484)

ROBERTS RUGH.*

From the Radiological Research Laboratory, Columbia University.

It has been established that ionizing radiations, whether from an x-ray source or from some isotope, may bring about the sterilization of animals if they are subjected either to large single or small chronic exposures(1-3, 6,8). Young mice of the C_{57} strain showed reduction in ovogenesis but no effect on spermatogenesis following injections of radioiodine (Gorbman—personal communications). Speert *et al.*(7) injected pregnant mice with radioiodine to determine the onset of fetal thyroid function, and found effects on the fetal gonad. Radioiodine readily passes from the nursing mouse to its litter young by way of the milk and there is a progressive increase in the relative amount of radioiodine previously injected into the nursing mouse, found in the suckling litter young. By 48 hours as much as 86% of the retained radioiodine is found in the litter members and only 14% in the mother(4,5).

It seemed of importance to determine the differential sensitivity of the ovary and the testes of the newborn mouse as it might be affected by radioiodine injected into the mother and transferred to the suckling young by way of the mammary glands.

Materials and method. The female mice used in this study were of the Swiss albino stock, each of which had previously delivered a normal litter. They were time-mated so as to litter simultaneously, and 37 litters were selected. The mice with small but normal litters were chosen so that there would be no milk shortage to the suckling young. In other words, there was available to them milk *ad libitum*. The 37 litters were treated as in Table I.

TABLE I.

Litters	Males	Females	Total	Post-partum age, days	I^{131} inj., μc
9	16	19	35	5	300
7	10	11	21	5	600
8	23	13	36	15	300
5	18	5	23	15	600
8	13	18	31	Controls	

The radioiodine was in the form of sodium iodide (NaI^{131}) received from the Pile at Oak Ridge and diluted so that each cubic centimeter contained 400 μc of active radioiodine. The dose levels used were 300 and 600 μc per single injection. The isotope was injected subcutaneously into the nursing mouse which was then returned to its litter. Sixteen mice were injected when the litter young were 5 days of age and 13 when their young were 15 days old. It is known that by the fifteenth day there is maximum milk production by the nursing mouse. Eight control females were injected with an amount of nonradioactive elementary iodine comparable to that of the higher dose levels of the isotope used. The shavings in all cages were discarded at 24 and 48 hours post injection to avoid any possible external contamination from excreted iodine and the mice were given the usual care of our animal quarters. At weaning time (4 weeks) the sexes were separated, members of one litter being kept separate from the members of other litters.

At 2 month intervals, beginning at 2 months of age and continuing until the mice were 10 months of age, about 10 mice from each of the 5 categories were selected at random and tested against normal stock for fertility. Each female was tested consecutively against 2 males for 5 days each, and each male was tested against 2 stock females. These same experimental mice were not again tested for fertility until all others, similarly treated, had been tested. At the termination of the experiment when the mice were 10 months of age, all living mice were fertility-tested.

Observations and experimental data. Table

---

* This study was undertaken with the assistance of a Grant from the Loyal League Philanthropies, and sponsored by the Committee on Tumor Registry of the American Academy of Pediatrics represented by Dr. H. A. Dargeon.

TABLE II. Fertility Tests.*

	I^{131} treatment of mother									
	5 days postpartum				15 days postpartum				Controls	
	300 μc		600 μc		300 μc		600 μc			
Age, mo.	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
2	5/6	4/4	5/5	4/5	2/5	5/5	5/5	6/6	3/3	5/5
4	3/4	2/4	3/4	1/4	4/5	5/5	5/5	4/6	6/6	4/4
6	5/5	2/5	3/4	0/5	5/5	2/5	5/5	3/6	6/6	5/6
8	4/4	3/4	4/4	0/4	4/4	4/5	3/3	3/6	3/3	4/4
Totals	17/19	11/17	15/17	5/17	15/19	16/20	18/18	16/18	18/18	18/19
%	90	65	88	29	79	80	100	89	100	95
Final test at 10 mo.	15/16	4/19	6/8	0/11	21/23	7/13	14/17	0/5	29/30	31/36
%	94	21	75	0	91	54	82	0	97	86

* The data are given in this manner: Fertile/total tested; hence fertility percentage can be determined directly.

II presents the fertility data of the entire experimental series. For practical reasons tests were not made on all animals every 2 months, but by random sampling and without duplication of animals until all had been tested, the data are considered to be representative.

With regard to the male offspring it is readily apparent that the fertility level is unaffected by any dose or time of treatment used. The lowest fertility value of 79% for males from 300 μc mothers injected on the 15th day is counterbalanced by the final test of the entire group of 23 animals which showed 91% fertility. The highest exposure of 600 μc I^{131} to the mother never reduced male offspring fertility below 75%. This figure was derived from the 10 month survivors of exposure on the 5th day postpartum. It is safe to conclude, therefore, that the male offspring subjected to radioiodine from the mother's milk is radioresistant with regard to its reproductive activity.

The data from the female offspring are, however, quite different. Sterility is evident as early as 4 months of age in mice exposed at 5 days post partem to either level of radioiodine injection of the mother. Those exposed at 15 days showed some sterilization by 4 months at the higher level and by 6 months at the lower level of injection of radioiodine. The overall fertility percentage for the period of 8 months indicates that the young ovary is more radiosensitive at 5 than at 15 days post partum. The smaller dose of 300 μc of radioiodine at 5 days reduced the fertility more (65% fertile) than the 600 μc dose at 15 days postpartum (89% fertile). This occurred in spite of the fact of greater milk production on the part of the mother. If one examines the final data of all remaining females at the termination of the experimental period, it is further evident that 600 μc of I^{131} to the mother at either the 5 or the 15 day postpartum period results in complete sterilization. This means that not only is the overall fertility reduced in the female, but the reproductive life span is likewise reduced. At the termination of the experiment 86% of the control females were still fertile.

Discussion. The reasons for the hypersensitivity of the young ovary as opposed to the young testes to the damaging effect of high and concentrated exposures to ionizing radiations from radioiodine have not been determined. It is very likely that the developing ovary and testes are not at the same stage of maturation at the time of radiation insult. However, this fact has been established for the fetal and adult mouse and the experimental evidence reported here substantiates the thesis for the newborn, receiving its exposure from maternal milk.

The question is raised with regard to a possible indirect effect on the suckling young when the mother is subjected to a large dose of radioiodine. There is no doubt but that either dose used would eventually ablate the mother's thyroid gland. Such radiation damage of the nursing mouse in no way alters her ability to suckle her young, to produce milk, or to survive for the duration of the experiment. The young mice are weaned at the same time as are the controls, and there is no outward evidence of functional or structural damage of any of the mice.

There is also the question of possible irradiation of the suckling young by the temporary radioactive mother. She certainly emits both beta and gamma radiations. This, no doubt, obtains for a very short period immediately after the injection but there is evidence that the relative amount of radioiodine shifts in favor of the litter young very rapidly (4,5). Further, even though the mother might be a considerable source of radiation for a short time, this does not detract from the thesis that the ovaries are more radiosensitive than are the testes. It would be difficult to postulate selective exposure of the sexes.

It has been shown(4) that the amount of I^{131} passing to any suckling litter member never exceeds 5% of the amount injected into the mother. This means that no more than 30 μc of I^{131} reached any of the young mice tested. Even this amount is rapidly reduced by excretion and decay. It can be stated, therefore, that the ovaries at 5 days appear to be more radiosensitive to an exposure of no more than 30 μc of I^{131} than do the ovaries at 15 days postpartum. This occurs in spite of the further fact that more milk is available to the suckling young on the 15th day.

Finally, the absolute amount of injected radioiodine into the adult has been shown to drop from 100% in 4 hours to 90% in 24 hours and 20% in 48 hours, due to excretion and to decay(4). If a comparable reduction rate occurs for the suckling young, the maximum concentration of 30 μc received by any suckling member was reduced in 48 hours to a maximum of 6 μc. The duration of irradiation of the tissues of the newborn mice is not long. Nevertheless, it seems evident that the gonads of the young newborn female mouse must be very sensitive to ionizing radiations from radioiodine, expressed in reduced fertility as early as four months of age.

Summary and conclusions. 1. Ovaries of young newborn mice are very sensitive to ionizing radiations from radioiodine administered to the mother and reaching the young through the milk. The testes of litter mate male mice are very radioresistant under the same conditions. 2. A single injection of a nursing mouse with 600 μc of I^{131} reduced fertility in the female offspring by four months and made all female offspring sterile by 10 months while controls were 86% fertile. Litter mate males were never reduced to less than 75% fertility. 3. It appears that the ovary of 5-day-old mice is more sensitive to ionizing radiations from radioiodine than is the ovary of the 15-day-old mouse. 4. Even though exposure of the ovary of the young mouse to ionizing radiations was relatively brief, sterility was not evident for at least 4 months. Not only was the overall fertility of the female reduced, but so also was the length of the reproductive life span.

---

1. Dunlap, C. E., *Human Fertility*, 1947, v12, 33.
2. Eschenbrenner, A. B., Miller, E., Lorenz, E., *Nat. Cancer Inst.*, 1948, v9, 3.
3. Hodges, P. C., *Postgrad. Med.*, 1952, v12, 152.
4. Rugh, R., *Morph.*, 1951, v89, 323.
5. ———, *Morph.*, 1951, v89, 457.
6. ———, *J. Exp. Zool.*, 1953, v122, 151.
7. Speert, H., Quimby, E., Werner, S. C., *Surg. Gyn. and Obst.*, 1951, v93, 230.
8. Warren, S., Dixon, F. J., *Radiology*, 1949, v52, 714.

Received June 12, 1953. P.S.E.B.M., 1953, v83.

Cholinesterase-Inhibiting Properties of Two Vinyl-Substituted Phosphates. (20485)

M. S. Morse, J. K. Kodama, and C. H. Hine.

From the Department of Pharmacology and Experimental Therapeutics, University of California School of Medicine, San Francisco.

Aromatic and halide derivatives of organo substituted phosphoric and phosphonic acids have found considerable use in agriculture and medicine. In general, their activity stems from their ability to combine with and inhibit the cholinesterases. We have studied a new series of organophosphorus compounds and have found certain unsaturated alkyl derivatives to be highly toxic to rodents(1). Corey and his associates(2) have reported the insecticidal activity of 2 of these, diethyl 2-chlorovinyl phosphate (I) and dimethyl 1-carbomethoxy-1-propen-2-yl phosphate (II). The report of Kodama(3) summarizes their mammalian toxicity.

In this paper we report on the cumulative inhibitory properties of I and II following repeated administration, the rate of regeneration or reactivation after single administration, and the inhibitory action *in vitro* of these compounds on cholinesterase. For comparative purposes, p-nitrophenyl diethyl thionophosphate (parathion) was included in the experimental design.

Method. Cumulative toxicity. Graded intraperitoneal injection. The cumulative inhibitory effect of different sublethal doses was determined by daily (except weekends) administration of each compound to female rats (100-156 g) of the Long-Evans strain. Groups of 10 rats received I, II, and parathion in amounts equal to 10, 25, and 50% of the calculated intraperitoneal LD_{50}. These LD_{50} values for female rats of this strain and weight range are, in mg/kg, for I, 9.0; for II, 1.51; and for parathion, 4.0. A group of 10 *control* rats received propylene glycol (the solvent used to facilitate administration to the experimental rats) in an amount equal to 0.2% of the body weight. Five rats from each group, or all survivors if there were fewer than 5, were sacrificed after 17 injections. The remaining rats were given a total of 22 injections before sacrifice. Twenty-four hours after their final injection, the rats were lightly anesthetized, and decapitated. The per cent of active cholinesterase present in the erythrocytes, plasma, and brain, was determined by a modification of the method of Frawley and his coworkers(4). Wherever advisable, cholinesterase values were examined by comparison of pH readings by means of the "Student" t test, or by analysis of variance. P values of greater than 0.05 were considered insignificant. *Varied number of injections.* The rate of cumulative inhibition was determined by repeated intraperitoneal administration of 25% of the LD_{50} of the compounds. Groups of 3 rats (110-140 g) were sacrificed 24 hours after 1, 2, 4, 8, and 16 daily injections. Sacrifice of the animals and evaluation of enzyme activity conformed with the procedure outlined above. *Sixty-day feeding.* Female rats (104-165 g) of the Long-Evans strain were divided randomly into groups of 6 and fed I and II at dietary levels of 6.3, 12.5, 25, 50, and 100 ppm. The phosphates were intimately mixed with a standard feed of known composition. A group of 10 rats served as control. At the end of 60 days, the surviving rats were anesthetized and decapitated, and the per cent activity of the cholinesterase in the erythrocytes, plasma, and brain, was determined.

Rate of regeneration. The rate of return of cholinesterase activity was determined in groups of rats (110-140 g) given 25% of the LD_{50} of I, II, and parathion, and sacrificed subsequently at appropriate time intervals until activity returned to normal. Five rats were used in each experimental group, and 5 in the control group. Groups were killed at ½, 1, 2, 4, 24, 48, and 96 hours after treatment, and in the case of I, also at 8 and 16 days. The cholinesterase content of erythrocytes, plasma, and brain, was determined for each rat according to the method of Frawley(4).

Inhibitory action in vitro. Inhibitory activity of the phosphates was determined *in vitro*, and the concentration at which the compound caused 50% inhibition of normal activity (IC_{50}) was determined for human erythrocytes, and for rat erythrocytes, brain, and plasma. The cholinesterase activity of human cells was determined by the method of Michel (5) while the activity of rat tissues was measured by Frawley's method.

Results. Cumulative toxicity. Graded intraperitoneal injection. There were no deaths in any of the groups receiving II, or in groups given I or parathion at the 10% level. Only one animal at the 25% level died, after 2 injections of I. Eight rats receiving 50% of the LD_{50} of I, and 8 at this level of parathion, died during the course of the experiment. There was a direct correlation between the degree of inactivation of erythrocyte and brain cholinesterase and the amount of inhibitor administered (Fig. 1). However, there were occasionally significant increases in plasma cholinesterase activity, *e.g.*, in animals receiving 10% of the LD_{50} of II and parathion. *Varying number of injections.* When 25% of the LD_{50} of the 3 phosphates was given for from one to 16 injections, the inhibition of erythrocyte cholinesterase by II was much less than that by the other phosphates, and values computed after the first, second, and fourth injections did not differ significantly from the control values. The cholinesterase activity of the erythrocytes fell progressively with increasing numbers of injections, of II and parathion; this effect was not evident with I, probably because of the original striking decrease to 20% of the control value after the first injection.

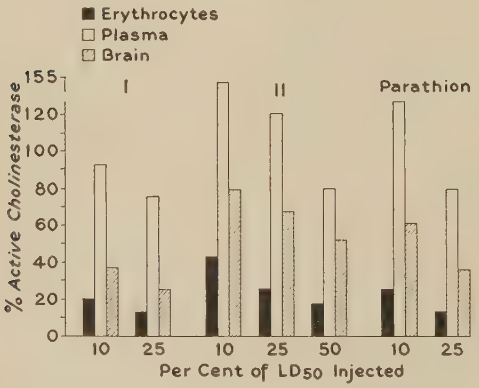


FIG. 1. % active cholinesterase of erythrocytes, plasma, and brain of female rats surviving 22 intraperitoneal injections of 10, 25, or 50% of the LD_{50} of I, II, or parathion.

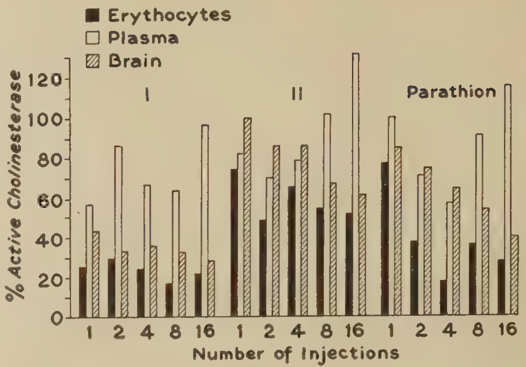


FIG. 2. % active cholinesterase in erythrocytes, plasma, and brain of female rats receiving 1 to 16 daily intraperitoneal injections of 25% of the LD_{50} of I, II, and parathion.

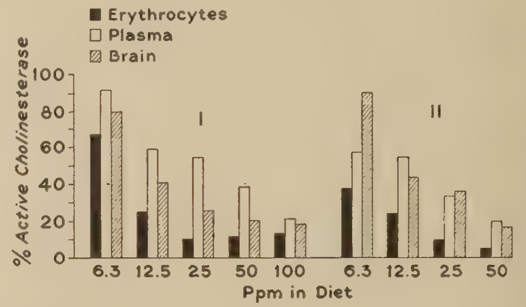


FIG. 3. % active cholinesterase in erythrocytes, plasma, and brain of female rats surviving diets containing 6.3, 12.5, 25, 50, or 100 ppm of I and II for 60 days.

There was no significant decrease in *brain cholinesterase* after the first injection of II and parathion, but all other values observed were significantly lower than those of control animals. The greatest depression followed I injections, and II had the least effect. A progressive decline in activity was seen with all 3 phosphates as the number of injections increased.

Plasma activity was not significantly decreased by II, and a significant increase appeared after the 16th injection. Plasma activity following parathion injection followed the same pattern, but with a greater initial depression, significant after the 4th injection. The subsequent rise was not significant by the

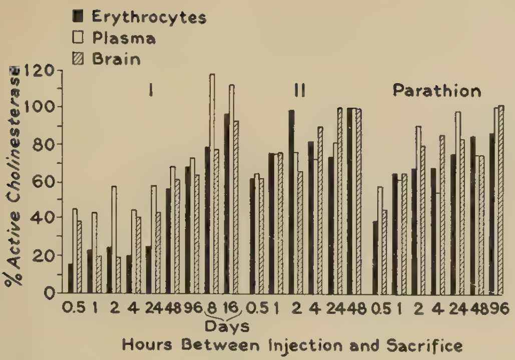


FIG. 4. % active cholinesterase in erythrocytes, plasma, and brain of female rats receiving one intraperitoneal injection of 25% of the LD_{50} of I, II, and parathion.

16th injection. Plasma activity was lowest after I injection, with a significant decrease after the first injection. There were no spontaneous deaths among the animals. Comparative cholinesterase values are shown in Fig. 2. *Subacute feeding.* No animals survived the feeding of II at a level of 100 ppm for 60 days, but 2 of the 6 fed 100 ppm of I survived. There were no deaths at lower levels. This experiment showed the most decided progressive decrease in cholinesterase activity correlated with increase in dosage, and all 3 tissues were affected (Fig. 3). The erythrocyte cholinesterase showed the greatest depression by both compounds. Brain cholinesterase was inhibited to the same extent by the 2 compounds, the only significant difference appearing at the 25% level, where II was less inhibitory ($p = 0.01$). Plasma cholinesterase showed the least inhibition.

Rate of regeneration. Cholinesterase activity of all tissues had returned to values not significantly different from control values by 24 hours after the injection of 25% of the LD_{50} of II and parathion. Depression was more severe and lasting with I. In general, the lowest values were observed within an hour after injection, and the rise thereafter was irregular rather than steadily progressive. The erythrocyte enzyme seemed to be inhibited to the greatest extent, and to regenerate a little more rapidly.

Inhibitory action in vitro. The results of the tests for activity *in vitro* did not follow the findings *in vivo*. Compound II was 10 times as active as I against all tissues studied. It was slightly more active than parathion against the enzyme of rat brain, however, and more active against rat plasma cholinesterase. The data are presented in Table I.

Discussion. The present studies indicated that I and II exhibit properties similar to the cholinesterase-inhibiting compound parathion. Inhibition of the activity of the cholinesterase of erythrocytes, brain, and plasma occurred to varying degrees, and was usually least and of shortest duration with II. Both vinyl phosphates and parathion showed greatest activity against the enzyme of erythrocytes, and least against the "pseudo" enzyme of plasma; the erythrocyte enzyme appeared to regenerate most rapidly. The plasma enzyme seemed to show a rise after the phosphates had been given intraperitoneally over a period of time, but not when fed. *Cholinesterase* inhibition by parathion resembled that of II after a single injection, but was more similar to that of I after multiple injections.

Close comparisons and absolute statements are difficult, because of the rather striking individual differences in the response of the rats to all 3 compounds, and the variations encountered in normal animals. Altered cholinesterase values were sometimes found after repeated injections of the supposedly innocuous propylene glycol, administered to control animals when used as a solvent for injection of experimental rats. The statistical analysis (analysis of variance and "Student" t test) presumably clarifies the data and enhances the reliability of our results.

In his studies of reversibility, DuBois and his coworkers(6) administered parathion intraperitoneally at a dose level of 5 mg/kg (71% of the stated LD_{50}), and determined that while the brain cholinesterase was depressed to 6% of the normal activity at one-half hour, it returned to 100% in 4 hours. Our experiment, using 25% of the calculated LD_{50}, showed less severe depression, to 57% of the control value. However, activity returned in the same length of time, 4 hours, to a value of 92% (not significantly different from the control value). These results compare satisfactorily, considering the comparatively small

TABLE I. Molar Concentration of Compounds I, II, and Parathion Required to Produce 50% Inhibition *In Vitro* of Cholinesterase from Various Sources.

Compound	Human erythrocytes	Rat erythrocytes	Rat brain	Rat plasma
I	2.1×10^{-5}	1.7×10^{-5}	1.1×10^{-5}	5.4×10^{-5}
II	2.4×10^{-6}	1.5×10^{-6}	2.0×10^{-6}	3.0×10^{-6}
Parathion	2.6×10^{-6}	3.2×10^{-6}	2.0×10^{-6}	3.0×10^{-6}

numbers of animals used and the unavoidable variations within groups.

Frawley(4), using the same dose as DuBois (16.6% of his stated LD_{50}) but peroral administration, found no inhibition at the end of 4 hours, and return to 90% of normal at the end of 14 hours. The values were no higher at the end of one week, and 2 weeks were required for the return of 100% activity of the brain enzyme. Lehman(7), on the other hand, using a peroral "sublethal" dose, found a maximum inhibition to 51%, and recovery in 72 hours. Erythrocyte cholinesterase in Lehman's experiment was reduced to 15% and did not return to normal for more than 4 weeks. According to Lehman, this prolonged recovery time shows that the enzyme-inhibitor complex is irreversible in erythrocytes, since the average life of a rat erythrocyte is 4 weeks. In our experiment, using intraperitoneal injection, recovery time was approximately 2 weeks, which, according to Lehman's theory, would seem to indicate reversibility.

Our results indicate that I and II resemble parathion more closely than DFP. The slower action and prolonged recovery time with I would suggest a slower absorption, transport, or metabolism.

Summary. 1. Comparative cholinesterase-inhibiting activity of 2 new vinyl-substituted phosphates and parathion was studied in respect to their cumulative inhibitory properties, the rate of regeneration of the enzyme following single administration, and their inhibitory action *in vitro*. 2. While qualitatively resembling parathion in inhibitory action against cholinesterase, quantitatively the diethyl 2-chlorovinyl phosphate was found to be more active both *in vitro* and *in vivo* than parathion; and the dimethyl 1-carbomethoxy-1-propen-2-yl phosphate was less active *in vivo*. 3. By examination of the cumulative inhibitory properties of these 3 phosphates, the observation of others has been confirmed, that on increasing the dosage or duration of exposure to the cholinesterase inhibitor, remarkably low levels of enzyme activity can occur without fatal termination. 4. Erythrocyte cholinesterase was usually depressed to a greater extent than that of brain or plasma. There was some indication of a tolerance developing on the part of the "pseudo" cholinesterase with one of the compounds.

---

1. Hine, C. H., Shack, E. R., Kodama, J. K., and Morse, M. S., *Fed. Proc.*, 1952, v11.

2. Corey, R. A., Dorman, S. C., Hall, W. E., Glover, L. C., and Whetstone, R. R., *Science*, in press.

3. Kodama, J. K., Morse, M. S., Hine, C. H., Anderson, H. H., and Dunlap, M. K., in press.

4. Frawley, J. P., Hagan, E. C., and Fitzhugh, O. G., *J. Pharmacol. and Exp. Therap.*, 1952, v105, 156.

5. Michel, H. O., *J. Lab. and Clin. Med.*, 1949, v34, 1564.

6. DuBois, K. P., Doull, J., Salerno, P. R., and Coon, J. M., *J. Pharmacol. and Exp. Therap.*, 1949, v95, 79.

7. Lehman, A. J., *Assoc. Food and Drug Officials U. S. Quart. Bull.*, 1952, v14, 85.

Received June 22, 1953. P.S.E.B.M., 1953, v83.

Effects of Hypophysectomy, ACTH, Cortisone and Estradiol on the β-Glucuronidase Activity of Adrenal Gland in the Rat.* (20486)

ERNEST KNOBIL.† (Introduced by Roy O. Greep.)
(With the technical assistance of Lorelei W. Parker.)
From the Biological Research Laboratory, Harvard School of Dental Medicine, Boston, Mass.

That a relationship exists between the activity of the enzyme β-glucuronidase in certain tissues and the circulating level of estrogens, androgens and probably adrenal cortical steroids is well established. Thus, estrogens notably increase the β-glucuronidase concentrations in the accessory organs of reproduction(1,2), androgens that in the kidney(3) and cortisone has been reported to effect a rise in the plasma levels of this enzyme in man(4). Although the nature of this hormone-enzyme relationship is still obscure, its elucidation may lead to a better understanding of the mechanisms involved in steroid hormone synthesis, utilization and metabolism. The occurrence of relatively high concentrations of β-glucuronidase in the adrenal glands of the rat(5) and the cow(6) seemed of interest in this regard and an investigation of adrenal β-glucuronidase activity under a variety of experimental conditions was therefore initiated.

Materials and methods. Male rats of the Sprague-Dawley strain were hypophysectomized by the parapharyngeal route, the control animals being subjected to sham procedures. At the time of operation 30,000 units of penicillin in oil were administered and 10% glucose was made available as the drinking fluid.

The 3 hormones were administered subcutaneously in one daily dose: estradiol benzoate in oil, cortisone acetate in aqueous suspension and ACTH in gelatin solution (ACTHAR gel Armour, Lot L-47704). The animals were sacrificed the day following the last injection of estradiol and cortisone and 3 hours after the last injection of ACTH.

The adrenals were trimmed, weighed on a torsion balance and analyzed for β-glucuronidase activity by the method of Talalay, Fishman and Huggins(5). In the hypophysectomized groups aliquots of the whole adrenal homogenates were utilized for the estimation of enzyme activity, total nitrogen determinations being performed on aliquots of the same homogenates by micro-Kjeldahl analysis.

Results. 1. *Effect of ACTH.* 2 mg of ACTH per day for 4 and 10 days effected a statistically significant increase in adrenal weight and treatment for 10 days resulted in a significant reduction of body weight (Table I). These expected changes were not associated with an increase in adrenal β-glucuronidase activity when expressed in terms of enzyme activity per mg of adrenal tissue, wet weight (Table I).

2. *Effect of cortisone acetate.* 3 mg of cortisone acetate per day for 10 days failed to effect a significant change in adrenal β-glucuronidase activity despite the marked regression in adrenal weight induced by this treatment (Table II).

3. *Effect of estradiol benzoate.* As in the case of ACTH and cortisone, estradiol (100 μg per day for 10 days) did not alter the β-glucuronidase concentrating adrenal tissue although the effects of this hormone on adrenal weight and somatic growth could be demonstrated. (Table II).

4. *Effect of hypophysectomy.* β-glucuronidase activity in the adrenal glands 6 days following hypophysectomy was increased significantly above the control concentrations ($P < .01$ in Exp. 1 and 2; Table III). When expressed in terms of enzyme activity per mg of nitrogen this increase was less marked but still significant at the 5% level (hypox.: 90.4 ± 7.9 units per mg of nitrogen; control: 70.3 ± 2.9 units per mg of nitrogen; 15 degrees of freedom).

Discussion. The increase in adrenal β-glucuronidase concentration following hypophysectomy is difficult to interpret. It should be noted that cortisone administration, while effecting an involution of the adrenals comparable to that obtained by hypophysectomy, did not result in a rise of enzyme activity.

---

* Aided by an Institutional Grant to Harvard University from the American Cancer Society.

† Milton Research Fellow, Harvard University.

TABLE I. Effect of ACTH on Adrenal β-glucuronidase Activity.

Exp.	No. animals	Final body wt	Adrenal wt	Units enzyme per mg adrenal*
		g	mg	
Control	6	195 ± 8†	25.4 ± 1.5	6.96 ± .51
2 mg ACTH/day for 4 days	7	186 ± 6	32.4 ± 2.1	6.70 ± .26
Control	5	228 ± 9	29.8 ± 2.5	7.92 ± .70
2 mg ACTH/day for 10 days	6	201 ± 1	41.2 ± 3.6	8.16 ± .28

* Wet wt.
† Mean ± stand. error of the mean.

TABLE II. Effect of Estradiol Benzoate and Cortisone Acetate on Adrenal β-glucuronidase Activity.

Exp.	No. animals	Final body wt	Adrenal wt	Units enzyme per mg adrenal*
		g	mg	
Control	9	206 ± 7†	26.3 ± 1.4	8.50 ± .54
100 μg estradiol benzoate/day for 10 days	10	152 ± 5	31.8 ± 2.0	7.97 ± .46
3 mg cortisone/day for 10 days	8	162 ± 8	12.8 ± .5	9.70 ± .61

* Wet wt.
† Mean ± stand. error of the mean.

TABLE III. Effect of Hypophysectomy (6 Days) on Adrenal β-glucuronidase Activity.

Exp.	No. animals	Final body wt	Adrenal wt	Units enzyme per mg adrenal*
		g	mg	
I				
Control	9	153 ± 3†	28.6 ± .7	10.28 ± .27
Hypox.	15	100 ± 3	15.9 ± .1	11.73 ± .35
II				
Control	10	164 ± 4	29.4 ± .8	10.59 ± .48
Hypox.	15	105 ± 3	16.1 ± .5	13.80 ± .37

* Wet wt.
† Mean ± stand. error of the mean.

Whatever the physiological significance of β-glucuronidase may be, its stability in the adrenal gland with reference to the experimental procedures employed suggests that the functions of the enzyme in this organ may be different from that in the uterus or the kidney where the response to hormonal stimulation or deprivation is a striking one.

Summary. The effects of hypophysectomy and ACTH, cortisone acetate and estradiol benzoate administration on the β-glucuronidase activity of the adrenal gland in the rat have been investigated. ACTH, cortisone and estradiol, while producing their characteristic effects on adrenal and body weights, failed to modify the β-glucuronidase concentrations in the gland. Hypophysectomy, on the other hand, effected a statistically significant increase in adrenal β-glucuronidase activity.

The estradiol benzoate was kindly supplied by Dr. P. L. Perlman, Schering Corporation, Bloomfield, N. J., and the cortisone acetate by Dr. E. Alpert of Merck and Co., Rahway, N. J.

1. Knobil, E., *Endocrinology,* 1952, v50, 16.
2. Leonard, S. L., and Knobil, E., *Endocrinology,* 1950, v47, 331.
3. Fishman, W. H., *Ann. N. Y. Acad. Sci.,* 1951, v54, 548.
4. Cohen, S. L., *Ann. N. Y. Acad. Sci.,* 1951, v54, 558.
5. Talalay, P., Fishman, W. H., and Huggins, C., *J. Biol. Chem.,* 1946, v66, 757.
6. Wattenberg, L. W., and Glick, D., *J. Histochem. and Cytochem.,* 1953, v1, 1.

Received June 24, 1953. P.S.E.B.M., 1953, v83.

Intracellular Phosphorus Turnover in the Rat Liver Cell.* (20487).

MARY GRIFFITHS AND NELLO PACE.
From the Department of Physiology, School of Medicine, and Office of Naval Research Unit No. 1, University of California, Berkeley.

With the advent of the technic of differential centrifugation for the preparation of large quantities of the sub-cellular particulates in isolated form, it has become possible to study quantitative aspects of the metabolic turnover of biochemical constituents at the level of the cell rather than at the tissue level. The present work represents an attempt to make a broad preliminary survey of the metabolic fate of radiophosphorus labeled phosphate in the rat liver cell following intravenous injection of the labeled phosphate.

Four intracellular fractions from rat liver have been investigated: mitochondria, microsomes, supernatant, and a crude nuclear fraction. The distribution of P32 in these fractions at different times after its injection into the animal as phosphate has been determined. As a preliminary step in the investigation of the individual substances in which P32 is turned over, the phosphorus compounds of each cell fraction have been chemically separated into the 4 following groups: the acid-soluble compounds, the phospholipids, the nucleic acids, and the protein residue. Thus it has been possible to construct time-distribution curves for labeled phosphorus in the various liver cell fractions, and within these, among the various chemical phosphorus fractions. Additional data has also been obtained on the dry weight, nitrogen content and total phosphorus content of the cell fractions from whole livers.

Experimental. Non-fasted male rats of the Curtis-Dunning strain weighing 250 to 300 g were used. For the preparation of the cellular fractions, livers previously perfused with cold Tyrode's solution were forced through a stainless steel sieve which retained the connective tissue, and the pulp was homogenized for 5 minutes in 0.88 molar sucrose in a glass homogenizer fitted with a water-cooled jacket. In general, 5 to 6 g of liver pulp were made up to a volume of 50 ml with sucrose.

Preparation of the cellular fractions. Essentially the method of Hogeboom *et al.*(1) was used for the differential centrifugation of the homogenate. The nuclear fraction was sedimented by centrifuging the homogenate twice for 15 and 10 minutes respectively at 600 x g in an "International" clinical centrifuge fitted with an angle-head rotor. The sediments from these 2 low-speed centrifugations were combined and washed twice with sucrose and constituted the washed crude nuclear fraction. The mitochondrial fraction was obtained by centrifuging the original supernatant removed from the nuclear fraction combined with the 2 washes, at 10,000 x g for 30 minutes in a "Servall" vacuum-type angle centrifuge. The sediment was washed once. The supernatant removed from the mitochondrial fraction, and from the subsequent washing of the mitochondria, was centrifuged for 30 minutes at 100,000 x g in a "Spinco" preparative centrifuge in order to sediment the microsomes. The microsomal fraction was washed once and resedimented. The entire operation of perfusion, homogenization and centrifugation was carried out in the cold at 2°C. Histological examination showed that the fractions obtained corresponded in appearance and staining properties with those described by Claude (2) and by Hogeboom and co-workers(1).

Dry weights of the cellular fractions. Modification of the above procedure was necessary in order to obtain the dry weights of the fractions free from sucrose. For each fraction, the initial centrifugation was carried out in sucrose, to ensure that the same fraction was being sedimented, but all washings were with 0.9% sodium chloride brought to a pH of 7.6 to minimize agglutination of granules. Sodium chloride was used rather than water because of the tendency for granules to disintegrate in water(2). No effort was made to allow for the small amount of sodium chloride con-

---

* These studies were aided by Contract N7 onr-29504 between the Office of Naval Research and the University of California.

TABLE I. Mean Values for Dry Weight, Nitrogen and Phosphorus Content of Cellular Fractions from 6 Rat Livers.

		Homogenate	Nuclei	Mitochondria	Microsomes	Supernatant	Recovery
Dry wt	Total g	2.37	.44	.75	.41	.77*	—
	% of total	100	18.7	31.0	17.4	32.5 *	
Nitrogen	Total mg	251.8	35.8	78.0	29.5	102.3	97.4
	% of total	100	14.3	31.0	11.7	40.4	
Phosphorus	Total mg	26.9	4.2	7.6	5.0	9.6	97.5
	% of total	100	15.6	28.3	18.7	34.9	
P/N ratio		.11	.12	.10	.17	.09	

* By difference, because of the presence of sucrose.

taminating the granules, which in no case amounted to more than 1 or 2% of the total dry weight of the fraction. The washed nuclei, mitochondria and microsomes were transferred to tared flasks and were dried to a constant weight at 90°C to 100°C. The dry weight of the supernatant was obtained by difference, because of the presence of the bulk of the sucrose in this fraction.

Nitrogen determinations. Nitrogen was determined by a micro-Kjeldahl method essentially as described by Umbreit(3). Steam distillation was carried out into a boric acid solution, using an indicator containing bromcresol green and methyl red, which gives a sharp end point.

Phosphorus determinations. The method of Sumner(4) was used for all phosphorus determinations. During digestion of the material with sulfuric acid and hydrogen peroxide, foaming due to large amounts of sugar present in some fractions was prevented or controlled by use of caprylic alcohol.

Analysis of the phosphorus compounds. The method of fractionation of phosphorus compounds into 4 main groups, proposed by Schneider was employed(5). Acid-soluble compounds were extracted with cold 10% trichloroacetic acid, followed by extraction of phospholipids with alcohol and ether, followed by extraction of nucleic acids with hot 5% trichloroacetic acid. The residue was dissolved in 1% sodium hydroxide and will be referred to as the protein residue. The phosphorus of each chemical fraction was determined as previously described. All samples were analyzed in duplicate.

P32 determinations. Approximately 20 μc (10^7 counts per minute) of high specific activity radioactive phosphorus in the form of NaH_2PO_4 were injected into the tail veins of the rats from 30 minutes to 48 hours before sacrificing. The 4 chemical fractions made from each cellular fraction were neutralized where necessary and placed on lens paper in the center of aluminum plates. All counting was done with a Geiger-Müller counter tube with a thin end window. Because this was a preliminary study designed only to indicate the overall picture of phosphorus exchange within the cell, no attempt was made to separate further the chemical fractions before determining the P32 content.

Results. Aliquots of the homogenate prepared from each of 6 rat livers were used for the dry weight, phosphorus and nitrogen determinations shown in Table I. The dry weight of the crude nuclear fraction varied from 14.9% to 22.1% of the dry weight of the homogenate, with a mean value of 18.7%. The mitochondrial fraction accounted for 27.4% to 35.0%, with a mean value of 31.5%, while the microsomes averaged 17.4% with a range of 14.7% to 21.0%. By difference, the supernatant was found to constitute a mean of 32.5% of the dry weight of the homogenate.

The mean phosphorus and nitrogen contents of the cellular fractions are also shown in Table I, both in terms of total milligrams present, and as percentages of the total phosphorus or nitrogen present in the liver. The mean phosphorus content of the whole liver, for this series of rats, was found to be 26.9 mg, 15.6% of which was in the crude nuclear fraction, 28.3% in the mitochondria, 18.7% in the

TABLE II. Phosphorus Distribution in Cellular Fractions of Rat Liver.*

	Homogenate	Nuclei	Mitochondria	Microsomes	Supernatant
Total P	30.11 (24.23-36.75)	4.60 (3.90-5.91)	6.89 (6.17-9.30)	6.13 (4.57-8.07)	12.01 (8.62-14.73)
Acid-soluble P	7.56 (5.96- 9.45)	.35 (.26- .50)	1.30 (1.08-1.62)	.11 (.07- .16)	5.82 (3.40- 7.63)
Phospholipid P	13.04 (10.40-16.50)	1.43 (1.14-1.85)	3.74 (3.20-5.25)	3.46 (2.50-4.32)	3.85 (2.94- 4.98)
Nucleic acid P	7.81 (6.38- 9.71)	2.41 (2.05-3.15)	1.28 (1.04-1.95)	2.26 (1.28-3.44)	1.88 (1.23- 2.66)
Protein residue P	1.23 (.91- 1.53)	.25 (.16- .33)	.51 (.41- .60)	.13 (.06- .23)	.24 (.11- .33)

* The values are means from 10 rats and are expressed as mg P per whole liver. The range is given in parenthesis below each mean.

TABLE III. Phosphorus of Chemical Fractions Expressed as % of Phosphorus of a Given Cellular Fraction.*

	Homogenate	Nuclei	Mitochondria	Microsomes	Supernatant
Total P, %	100	100	100	100	100
Acid-soluble P, %	25.1	7.6	18.9	1.8	48.5
Phospholipid P, %	43.3	31.1	54.3	56.4	32.1
Nucleic acid P, %	25.9	52.4	18.6	36.9	15.7
Protein residue P, %	4.1	5.4	7.4	2.1	2.0

* Mean values from 10 rats.

microsomes, and 34.9% in the supernatant. The same livers contained, on an average, 251.8 mg of nitrogen, 14.3% of which was in the nuclei, 31.0% in the mitochondria, 11.7% in the microsomes, and 40.4% in the supernatant.

Tables II and III show the intracellular distribution of phosphorus in the livers of a second series of rats. In Table II, the total phosphorus content of the cellular fractions is given in mg and this is broken down between the different chemical fractions. The mean and range for 10 rats are given. Although the wet weight of the livers of the 10 rats varied not very greatly, the phosphorus content ranged from 24.2 mg to 36.7 mg per liver. Where the phosphorus content was low the fraction most affected was invariably the supernatant. Comparison of Tables I and II shows that the mean values for the phosphorus contents of the cellular fractions were not exactly the same for the two series of rats; however, the differences between the means were not statistically significant by the *t* test.

In Table III, the chemical form in which the phosphorus is present in each cellular fraction is stressed by expressing the phosphorus content of a given chemical fraction as a percentage of the total phosphorus of the cellular fraction from which it was obtained. This brings out more clearly several points. Each cellular fraction, including the supernatant, contains a high percentage of phospholipids, which in the case of the mitochondria and microsomes amounts to more than 50% of the total phosphorus present. The supernatant contains a very high percentage of acid-soluble phosphorus, which might be expected, but the microsomes contain extremely little (1.8% of their total phosphorus), as compared with the mitochondria in which 18.9% of the total phosphorus is acid-soluble. The reverse is true of the nucleic acid content, where nucleic acid phosphorus accounts for 36.9% of the phosphorus of the microsomes but only 18.6% of the phosphorus of the mitochondria. Protein residue is low in all fractions. It averages 7.4% of the total phosphorus in the mitochondria but only 2.1% and 2.0% in the microsomes and supernatant respectively.[†]

Uptake of P32 by the cellular fractions.

[†] The amount of protein residue phosphorus obtained depends upon the method of extraction used. The Schneider method used here gives values 10 times greater than those obtained by the Schmidt-Thannhauser method(6).

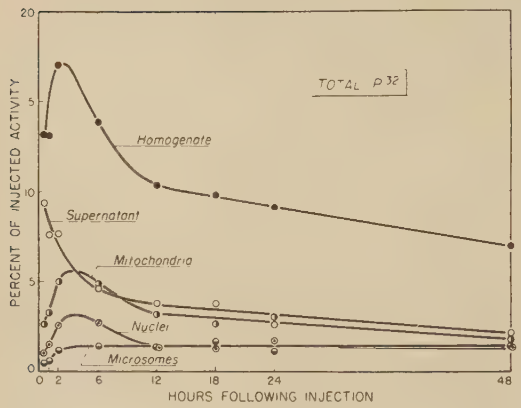


FIG. 1. Distribution of total P32 activity in various fractions of rat liver cells as a function of time following injection.

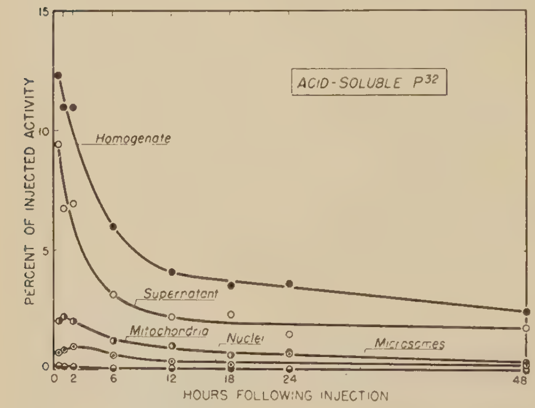


FIG. 2. Distribution of acid-soluble P32 activity in various fractions of rat liver cells as a function of time following injection.

Fig. 1 to 4 show the % distribution of the total injected dose of P32 in the cellular fractions of the rat liver one-half hour to 48 hours after intravenous injection of P32. Each point represents the mean value of 2 rats. From Fig. 1 it appears that the P32 reaches a maximum in the liver as a whole about 2 hours after intravenous injection. In the first few hours there is a similarity in the curves of the mitochondrial and crude nuclear fractions, which both take up P32 rapidly and reach a maximum at about 4 hours after injection. The microsomes on the other hand take up relatively little P32, very slowly. The P32 uptake of the supernatant follows an entirely different course, reaching a maximum some time before one-half hour after injection, and dropping off very rapidly at a time when the P32 is being incorporated most rapidly into the particles.

The incorporation of P32 into the acid-soluble compounds of the cellular fractions is shown in Fig. 2. This includes both inorganic and esterified phosphate compounds. Quantitatively, the supernatant is clearly the most important component at all times, while the nuclei and particularly the microsomes are relatively unimportant.

Fig. 3 gives the distribution of phospholipid P32 among the cellular components. The most noticeable features are the similarity of the curves for all fractions including the supernatant, the maximum occurring in all cases about 6 hours after injection.

The distribution of nucleic acid P32 is

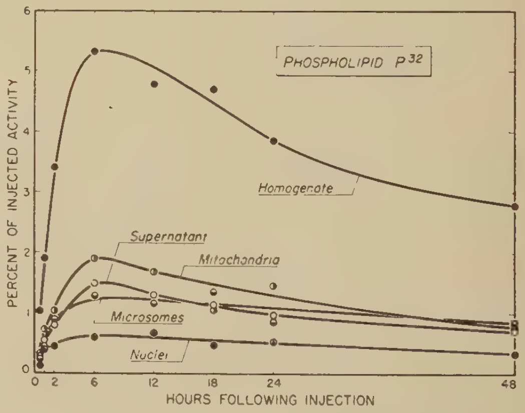


FIG. 3. Distribution of phospholipid P32 activity in various fractions of rat liver cells as a function of time following injection.

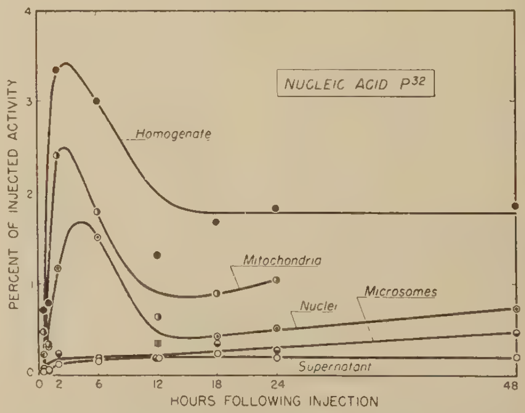


FIG. 4. Distribution of nucleic acid P32 activity in various fractions of rat liver cells as a function of time following injection.

given in Fig. 4. This fraction sometimes showed considerable variation between 2 rats sacrificed at the same time, and in general seems to be a less constant component than the phospholipids or acid-soluble compounds. Initially, only a very small amount of P32 is found in the nucleic acids of the microsomes and supernatant compared with the nuclei and mitochondria. Incorporation into the mitochondrial fraction is the most rapid, a maximum being reached 2 to 3 hours after injection, as compared with 5 to 6 hours for the nuclei, and considerably longer for the microsomes and supernatant.

Data for the protein residue are not included because the amounts of radioactivity and phosphorus were in some cases so small that accurate determinations were not feasible. However, the high specific activity that has frequently been reported(7) when the Schmidt-Thannhauser method of separation is used was not found in this investigation where the Schneider extraction method was employed. The role of this class of compounds in the metabolism of the cell will remain obscure until their chemical nature is more rigorously defined.

Discussion. There is considerable variation in the dry weights of all cellular fractions. This is particularly noticeable in the crude nuclear fraction, where it can be ascribed in part to deficiencies in the separation procedure, since this fraction is not pure and may well contain varying numbers of unbroken cells. Nuclear volumes from 10 to 18% of the liver cell volume have been reported(8) so the dry weight figure of 18.7% of the dry weight of the liver is probably not much too high. Although the nuclear fraction is admittedly impure, nevertheless its properties are probably largely those of pure nuclei. The results with this fraction are included for general comparative purposes, and to complete the quantitative recovery figures.

The situation is somewhat different with regard to the mitochondria. Losses were kept to a minimum by reclaiming mitochondria from the nuclear washes, and although Jackson *et al.*(9) have found that repeated washing is necessary to rid the mitochondria of all sub-microscopic particles, the degree of contamination involved here is not sufficiently great to account for the observed variations in dry weights. Many conditions such as nutritional status(10), or glycogen-glucose balance(11), have been shown to influence the state or number of the mitochondria of various cells, and this would clearly be reflected in the dry weight.

Greater variation was found in the dry weights of the microsomes than of the mitochondria. The rats used in this experiment were non-fasted as this was considered preferable for the subsequent experiment where the animals were sacrificed from one-half hour to 48 hours after the injection of P32. Changes in liver metabolism, for example the utilization of glucose, have been shown to occur with prolonged fasting(12). However the disadvantage of using non-fasted rats is that glycogen granules are present in the liver and these appeared largely in the microsome fraction. Variation in the amount of glycogen present greatly affected the dry weight of this fraction. Another experiment in which fasted rats were used gave much more consistent and considerably lower values for the dry weight of the microsome fraction.

In the crude nuclear fraction the nitrogen content was 14.3% of the total nitrogen of the homogenate, which is in quite close agreement with Schneider's finding of 13.3%(13). Many figures are available for the nitrogen content of the mitochondria and they range between 23% and 26% of the total nitrogen. However, after correcting for losses, Schneider and Hogeboom(14) have estimated that 30 to 35% of the total nitrogen is present in this fraction. Their estimate agrees well with the value of 31% found in this investigation, where losses were kept to a minimum.

The nitrogen and phosphorus values for the microsomes reported here are considerably lower than those found in some investigations where centrifugation was more prolonged and smaller particles were sedimented(1). The dry weight of this fraction can clearly be increased by increasing the time or speed of centrifugation. This acceleration of 100,000 x g for 30 minutes used in the present experiments was somewhat arbitrarily chosen, in order to reduce the time of separation to a reasonable minimum. On the other hand, the high phosphorus to nitrogen ratio, which is characteristic of microsomes, was obtained, being 0.17 for the microsomes as compared with 0.10 for the mitochondria and 0.09 for the supernatant.

The data presented in Tables II and III reveal a number of points concerning the partition of phosphorus compounds between the cellular components. It is not surprising that there is a large amount of acid-soluble phosphorus present in the supernatant, as it would be expected that a wide variety of relatively small, phosphorus-containing molecules is present in the non-granular part of the cytoplasm. But the very large amount of these substances present in the mitochondria is striking, especially when compared with the extremely small amount associated with the microsomes. It may be that the presence of semi-permeable membranes around the mitochondria permits these granules to retain acid-soluble substances where no such mechanism exists in the smaller granules. It was noticed that the acid-soluble extract of the mitochondria was quite green compared with other fractions; possibly they are responsible for the elaboration of some of the constituents of bile.

It can be seen from Table I that phospholipids constitute the major group of phosphorus-containing compounds in the liver cell, accounting for 43% of the total phosphorus. They are more concentrated in the granules than in the nuclei or cytoplasm, but, unlike the acid-soluble compounds they occur in quantity in every cellular component.

There has been some question as to whether all the nucleic acid of the cell is bound to particles, or whether some is free in the supernatant. Table I shows that a considerable amount of nucleic acid, 24%, is not sedimented by centrifugation at 100,000 x g for 30 minutes, and several investigators have confirmed the presence of nucleic acid which is nonsedimentable at even higher speeds(14, 15). However, Szafarz(16) has shown that this nucleic acid does not exist in the supernatant in the form of relatively small molecules, but is bound to proteins by stable bonds analogous to those existing in mitochondria and microsomes.

A large number of somewhat conflicting estimates are given in the literature for the nucleic acid content of cell particles(14). The data presented in Table II support the general view that the highest concentration of nucleic acid occurs in the microsomes, with considerably lower values for the mitochondria and supernatant.

Considering the more dynamic aspect of phosphorus distribution, Fig. 1 to 4, showing the incorporation of P32 into the cellular components, reveal several interesting points. Because the purpose of this investigation was to make a broad survey of the outlines of phosphorus metabolism within the cell, rather than an attempt to establish in accurate detail the turnover of any one class of compounds, only the general nature of the curves obtained should be considered. Furthermore, no attempt was made, at this time, to separate inorganic phosphate from the remainder of the acid-soluble fraction. Therefore the activity of this fraction represents not only the incorporation of P32 into esterified compounds, but also the penetration into the cell fractions of the original injected phosphate. However, it appears that after the first 2 or 3 hours, the bulk of the acid-soluble P32 is in a bound form, as the specific activity of the supernatant acid-soluble phosphorus is, by that time, lower than that of any other fraction.

Labeled phosphorus enters the liver cell very soon after injection and the activity of the phosphorus of the non-particulate cytoplasm rises immediately. Incorporation of P32 into the mitochondria and nuclei follows rapidly, but proceeds more slowly in the microsomes. The specific activity of the phosphorus of the microsomes was at all times lower than that of the other fractions. This rapid incorporation of P32 into the mitochondria as compared with the microsomes, is in keeping with the many metabolic activities ascribed to them. There is a great deal of evidence that much of the production of energy takes place at the level of the mitochondria. Here the various reactions of the Krebs cycle occur, with the production of many phosphorylated intermediates(17,18); and it is probably also in the mitochondria that this energy is made available to the rest of the cell by the production of high energy phosphates(14). It is therefore not surprising to find that the incorporation of P32 into acid-soluble compounds is particularly extensive in the mitochondria.

In contrast to the behavior of the mitochondria is the relatively slight incorporation of P32 by the microsomes: particles known to have very incomplete enzyme systems and to function to only a minor extent in the oxidative processes of the cell. This difference in activity of the 2 particles is yet another indication that the 2 types of particles exist in the cell, and that the microsomes obtained by differential centrifugation are not merely broken up mitochondria, or the mitochondria simply aggregates of microsomes.

The acid-soluble phosphorus of the crude nuclei presents a problem requiring further investigation. Preliminary study of the specific activities of the phosphorus of the acid-soluble fractions has shown that the specific activity of the acid-soluble phosphorus of the crude nuclei appears to be higher than that of any other cellular fraction, in some cases being as much as 5 times greater, and therefore cannot represent merely contamination by other cellular constituents. The acid soluble phosphorus compounds of the nuclei are apparently metabolically very active, even though small in amount. Perhaps they contain an exceptionally high proportion of substances such as adenosine triphosphate, which is known to have a rapid turnover in the liver (19). It will be necessary to use pure nuclear preparations and to separate out the various known components of the acid-soluble fraction in order to determine the compound or compounds involved.

The large amount of phospholipid present in each cellular fraction and the extreme similarity with regard to the incorporation of P32 into phospholipids in all parts of the cell is very striking, and would tend to favor a theory of the structural function of phospholipids in the cell. This is in marked contrast to the nucleic acids which vary greatly in their turnover, depending upon their location within the cell.

Summary. 1. A broad preliminary survey has been made of the metabolic fate of P32 labeled phosphate within the rat liver cell, following intravenous injection of the labeled phosphate. 2. By means of differential centrifugation of rat liver homogenates, 4 intracellular components have been separated out: mitochondria, microsomes, the remaining supernatant, and a crude nuclear fraction. 3. Time-distribution curves have been constructed for the P32 in the 4 cell fractions, and further resolution has been obtained by chemically fractionating the P32 activity in each cell fraction into 4 groups of phosphorus compounds: the acid-soluble compounds, phospholipids, nucleic acids, and protein residue. 4. The mitochondria and nuclei took up P32 rapidly, whereas the microsomes incorporated relatively little P32, and much more slowly. Characteristically different patterns of P32 uptake were also noted for the various chemical fractions. A brief interpretation of the findings is given. 5. Additional data are given for the dry weight, phosphorus and nitrogen content of the cellular fractions from whole rat livers.

---

1. Hogeboom, G. H., Schneider, W. C., and Palade, G. E., *J. Biol. Chem.*, 1948, v172, 619.

2. Claude, A., *J. Exp. Med.*, 1946, v84, 51.

3. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Tissue Metabolism*, Minneapolis, 1949.

4. Sumner, J. B., *Science*, 1944, v100, 413.

5. Schneider, W. C., *J. Biol. Chem.*, 1945, v161, 293.

6. Davidson, J. N., *The Biochemistry of the Nucleic Acids*, John Wiley and Sons, New York, 1950, p61.

7. Davidson, J. N., Gardner, M., Hutchison, W. C., McIndoe, W. M., Raymond, W. H. A., and Shaw, J. F., *Biochem. J.*, 1949, v44, xx.

8. Mirsky, A. E., and Ris, H., *Nature*, 1949, v163, 666.

9. Jackson, K. L., Walker, E. L., and Pace, N., *Science*, 1953, v118, 136.

10. Lagerstedt, S., *Exp. Cell. Research, Suppl.*, 1949, v1, 380.

11. Kater, J. M, *Anat. Rec.*, 1931, v49, 277.

12. Tomkins, G. M., and Chaikoff, I. L., *J. Biol. Chem.*, 1952, v196, 569.

13. Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, 1950, v183, 123.

14. ———, *Cancer Research*, 1951, v11, 1.

15. Huseby, R. A., and Barnum, C. P., *Arch. Biochem.*, 1950, v26, 187.

16. Szafarz, D., *Biochim. et Biophys. Acta*, 1951, v6, 562.

17. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1949, v177, 893.

18. Kennedy, E. P., and Lehninger, A. L., *J. Biol. Chem.*, 1949, v179, 957.

19. Hevesy, G., *Radioactive Indicators*, Interscience Publishers, Inc., New York, 1948.

Received June 24, 1953. P.S.E.B.M., 1953, v83.

Virulence Enhancement of *Candida albicans* by Antibiotics and Cortisone. (20488)

ERICH SELIGMANN. (Introduced by W. Antopol.)

From the Division of Laboratories, Beth Israel Hospital, New York and the Joseph and Helen Yeamans Levy Foundation.

It was previously reported that the intraperitoneal injection of a non-pathogenic strain of *Candida albicans* produces a rapidly fatal disseminated infection if aureomycin is administered simultaneously by the same route (1). That this effect was not due to this special strain of Candida is evident from the fact that similar results were obtained with 9 other strains of Candida, 2 isolated from the oral cavity, 4 from urine, and 3 from stool of patients. With the exception of minor quantitative differences, the results with these strains were similar to those reported in the original communication. The death ratio varied from 60-100%; with an average of 76.6% (83 deaths among 107 mice). None of these strains alone was capable of causing death of the mice without concomitant intraperitoneal administration of aureomycin.

Routes of administration of aureomycin and fungus. When the drug was administered orally (2 mg aureomycin in 0.2 cc saline), and the fungus was given *per os*, subcutaneously or intraperitoneally, no death occurred; after the subcutaneous injection of aureomycin and oral or intraperitoneal infection, no deaths occurred; when the drug and fungus were mixed and given subcutaneously 2 out of 10 animals succumbed; after the intraperitoneal injection of aureomycin, oral and subcutaneous infection proved harmless. However, a mixture of drug and fungus administered intraperitoneally invariably proved fatal (Table I).

TABLE I. Various Routes of Drug and Fungus Administration.

Application of drug	Infection with Candida	Results with— Aureomycin	Terramycin
Per os	*Per os*	0/10	0/10
	Subcut.	0/10	0/10
	Intraper.	0/9	0/10
Subcut.	*Per os*	0/10	1/10
	Subcut.	2/10	4/10
	Intraper.	1/10	4/10
Intraper.	*Per os*	0/10	1/10
	Subcut.	1/10	2/10
	Intraper.	10/10	10/10

Legend: Doses of aureomycin, 2 mg in 0.2 ml; of terramycin, 3.3 mg in 0.2 ml. Doses of *Candida albicans*, 0.2 ml of an emulsion of packed cells in 2.5 ml saline (320 million living cells).

Numerator = Mice dead within 14 days of observation. Denominator = Total No. of mice.

All of 6 mice survived the intravenous administration of 1 mg aureomycin in 0.1 ml saline; 21 of 30 mice succumbed after the intravenous administration of 30, 40, or 80 million Candida cells in 0.1 ml saline, but only 5 of 21 mice succumbed when the same number of Candida cells were suspended in 0.1 ml (1.0 mg) of aureomycin. It is evident that intravenously introduced *Candida albicans* is pathogenic for mice and that the pathogenic effect of the fungus is remarkably reduced when combined with aureomycin. These results contrast with all other combinations of routes of administration.

Tests with other antibiotics. In another series of experiments other antibiotics were employed in place of aureomycin. The test (simultaneous injection of Candida and the antibiotic intraperitoneally) was performed with penicillin G (up to 10,000 units in 0.2 ml saline), streptomycin (10 mg), bacitracin (up to 200 units), and chloromycetin (up to 5 mg in 0.2 ml solution) in propylenglycol, 50% acetamide or 50% N,N-dimethylacetamide. The aureomycin results were not duplicated. Some animals succumbed in the course of the experiment, but most of the deaths were late occurring from 7 to 14 days after treatment. These findings could not be compared with the immediate action of aureomycin. Of 54 mice treated with the various antibiotics 18 died, 12 in this belated fashion. The recovery of Candida from the organs of several of these cases indicates that some interaction between drugs and fungus may have taken place. In control experiments 12 mice received 0.2 ml Candida emulsion intraperitoneally and no drug. The animals were sacrificed after 1, 5, 9, 10, and 14 days. Cultures from kidneys were all negative for Candida. The yeast cells obviously were excreted under these "normal" conditions with no permanent damage to the renal tissue.

*Terramycin** was the only antibiotic that simulated the action of aureomycin. A dose of 2 mg was insufficient to produce constant results; but amounts of 3.3 or 5 mg in 0.2 ml saline solution were fully as effective as aureomycin. The technic previously described(1) was employed. With intraperitoneal injection of terramycin-Candida mixture, 26 out of 27 experimental animals died within 24-48 hours with a dose of 5 mg terramycin, and 19 out of 23 died when 3.3 mg terramycin was used. The terramycin solutions in these tests were standing from one to 3 days after preparation. No difference was found with freshly prepared solutions as compared with 3-day-old solutions, indicating a stability of the virulence enhancing factor which corresponds to the antibiotic stability(2,3). This contrasts with aureomycin which is much less stable when assayed for antibiotic activity as well as in the drug-fungus test; on standing the virulence enhancing and antibiotic capacities of aureomycin were lost after 1 or 2 days. Boiling, which destroys both these qualities of aureomycin, does not alter these same effects of terramycin. All of 31 animals succumbed after receiving boiled terramycin mixed with fungus. Numerous subcultures on Sabouraud's agar were made from the heart blood and from the kidneys of dead mice. Candida was recovered in 26 out of 27 kidney cultures and in 13 out of 22 heart blood cultures, indicating that a generalization of the monilia infection had taken place within the body of the antibiotic treated animals. Frequently grossly visible abscesses were observed in the kidneys.

Various routes of drug and fungus application. The various routes of administration of terramycin and *Candida albicans* in mice were tested as described for aureomycin in this communication. Terramycin appears to be slightly more active than aureomycin, as can be seen from Table I, but the overall results with both antibiotics are essentially alike: early death when drug and fungus are given intraperitoneally, and none or little action under other conditions (Table I). *Intravenous injection.* Results simulate those observed with aureomycin. Of 10 animals injected with 80 million yeast cells in 0.1 ml saline, one survived. Of 10 mice injected with 0.1 ml (1.7 mg) terramycin solution, 9 survived. Of 10 mice receiving 80 million Candida cells in terramycin (1.7 mg) 3 survived. The time factor in the occurrence of deaths may be seen from Table II. Thus terramycin as well as aureomycin reduce the pathogenicity of Candida on intravenous injection of the drug-fungus mixture, in contrast to all other forms of application.

Cortisone. It has been reported that this steroid exerts a definite influence on induced infection in mice, whether the infecting agent be either bacteria(4) or viruses(5). Dormant infections became manifest or sublethal doses developed fatal effects(6). Since these ob-

---

* Crystalline Terramycin hydrochloride intravenous was used. It is a yellow powder in mixture with sodium glycinate which, according to the C. Pfizer and Co. statement, retains its antibiotic potency for at least 12 months at room temperature. It is readily soluble in water and saline solution: this solution is undiminished in antibiotic activity after storage in the refrigerator for at least 3 days.

TABLE II. Result of Intravenous Injections.

	No. of	Surviving .... days after													
Material injected	mice	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0.1 ml Candida emulsion (80 million cells)	10	7	3	3	1	1	1	1	—	—	—	—	—	—	—
0.1 ml terramycin solution (1.7 mg)	10	10	10	10	10	10	10	10	10	10	10	9	9	9	9
0.1 ml terramycin-Candida mixture	10	8	7	7	6	6	5	5	5	5	5	5	4	4	3

servations are paralleled by our results with antibiotics and *Candida albicans*, similar experiments were set up with cortisone and Candida infection.

Intraperitoneal injection of Candida emulsion in cortisone (2.5 mg) as used in the antibiotic tests proved harmless; none of 7 treated mice became sick. When 5 mg cortisone was given subcutaneously and the fungus intraperitoneally at the same time, 3 out of 10 animals died. The kidneys of all 3 yielded *Candida albicans* cultures, one heart blood culture also was positive. In further experiments 2.5 mg cortisone was given subcutaneously 2 hours before fungus infection (2.5 mg intraperitoneally) and 2 hours after the infection, with striking results: 21 of 22 animals succumbed in the course of the 2 weeks following treatment. On autopsy numerous abscesses were found in all the internal organs, particularly liver, spleen, omentum, and kidneys. The latter organ was most heavily infected, being studded with numerous small abscesses. In 19 of the 21 cases *Candida albicans* was recovered from the kidneys, in 14 from heart blood.

Discussion. Previous experiments demonstrating virulence enhancing qualities of aureomycin on Candida were continued and expanded. Nine more strains of *Candida albicans* isolated from human sources reacted with aureomycin in the same manner as the original strain. Of the other antibiotics tested in the drug-fungus combination, only terramycin gave similar results. The similarity of action of the 2 drugs in growth inhibition and growth promotion in animals is well known (7). Their chemical structure, although different in details, shows a similar basic structure(8).

Virulence enhancing capacity of terramycin closely parallels its antibiotic activities. In contrast to the more labile aureomycin, terramycin is more stable and the virulence enhancing and antibiotic activity is retained after standing or boiling. Various routes of administration of drug and fungus produce similar results with both antibiotics. In both the intravenous route of drug-fungus injection did not lead to virulence enhancement, but on the contrary had some antifungal effect.

That cortisone lowers resistance in mice has been described repeatedly. This steroid, therefore, was tested in *Candida albicans* infected mice. When given subcutaneously in large amounts (2.5 mg) before and after i.p. fungus infection, death resulted regularly. A less rapidly fatal infection developed due to the invasion of the blood stream, with widely disseminated abscesses, particularly in the kidneys. *Candida albicans* was regularly found in pure culture from kidneys and frequently from the heart of the dead mice. Material taken from abscesses in spleen and liver likewise contained Candida organisms only. No gram positive rods (Corynebacterium) or gram negative organisms (salmonella) nor staphylococci were found in any of these organs.

In this and in the preceding communication(1) the term "virulence enhancement" has been used to indicate the increased morbidity and mortality. Perhaps this is a misnomer since virulence is not always a single quality, but represents the result of the aggressive forces of the infecting agent and the defensive power of the infected host. Therefore, enhancement of virulence may be conditioned by increased aggressiveness of the fungus or by modified resistance of the mouse. From the findings herein described it appears that aureomycin, terramycin, and cortisone lower resistance of the mouse to infection. The same holds true for the action of mucin (9). No evidence could be found indicating a direct influence of the antibiotics on the fungus either *in vitro* or *in vivo*, pointing to a modification of the host's cell.

Summary. 1. Terramycin has the same virulence enhancing effect on *Candida albicans* as aureomycin. Other antibiotics failed to show this effect. 2. Terramycin due to its stability acts after standing or boiling while aureomycin, which is not stable on standing and is heat labile, does not. This parallels their antibiotic activities. 3. Only the intraperitoneal injection of either drug and fungus is effective in producing a fatal infection. All other routes of administration and combination were negative. 4. Intravenous injection of drug and fungus indicates a protective effect. 5. Cortisone administered shortly before and after infection with *Candida albicans* produces a generalized fatal infection. 6. Lowered resistance of the animal to infection is considered as the cause of "virulence enhancement."

1. Seligmann, E., Proc. Soc. Exp. Biol. and Med., 1952, v79, 481.
2. Regna, P. P., and Solomons, I. A., *Ann. New York Acad. Sci.*, 1950, v53, 229.
3. Gocke, T. M., Jackson, G. C., Wilcox, C., and Finland, M., *Ann. New York Acad. Sci.*, 1950, v53, 297.
4. Antopol, W., Glaubach, S., and Quittner, H., *Rheumatism (London)* 1951, v7, 187.
5. Southam, C. M., and Babcock, V., Proc. Soc. Exp. Biol. and Med., 1951, v78, 105.
6. Antopol, W., Proc. Soc. Exp. Biol. and Med., 1950, v73, 262.
7. Hobby, G., *Bact. Rev.*, 1953, v17, 36.
8. Regna, P. P., *Transact. New York Acad. Sci.*, 1952, Ser. II, v15, 12.
9. Olitzki, L., *Bact. Rev.*, 1948, v12, 149.

Received June 25, 1953. P.S.E.B.M., 1953, v83.

Effect of Anterior Pituitary Growth Hormone on Certain Liver Enzymes. (20489)

G. H. Beaton, G. Ozawa, J. R. Beaton, and E. W. McHenry.
(Introduced by R. C. Parker.)

From the Department of Public Health Nutrition, University of Toronto, Toronto, Canada.

It has been reported(1) that growth hormone decreases the catabolism and excretion of amino acids rather than directly affecting the synthesis of body protein. In contrast, Russell(2) using nephrectomized rats given casein hydrolysate, found that the principal action of growth hormone is to increase amino acid uptake into tissues. This agrees with the results of Friedberg and Greenberg(3) who found an increased incorporation of isotopic methionine into skeletal muscle following growth hormone administration. Bartlett and Glynn(4) observed no effect of growth hormone on liver or renal aspartic-glutamic transaminase in the normal adult female rat though the hormone increased liver aspartic-glutamic transaminase in immature hypophysectomized rats. Fraenkel-Conrat *et al.*(5) have shown that the administration of growth hormone to plateaued female rats for 8 days at a level of 3 mg per day resulted in decreased fasting arginase activity in pooled liver samples. It seemed advisable to study the effect of growth hormone, given in acute dosage to normal rats, on the activities of certain liver enzymes involved in nitrogen metabolism.

Methods and results. Eighteen adult male rats of the Wistar strain which had been maintained on a stock chow diet and water *ad libitum* were divided into 2 groups of comparable average body weight (380 g; range 322 to 429 g). Following a fast of 22 hours, one group was injected intraperitoneally with an anterior pituitary growth hormone preparation (Connaught Medical Research Laboratories, Lot 200) in saline (made slightly alkaline) at a level of 3 mg per 100 g body

TABLE I. Effects of Anterior Pituitary Growth Hormone on Liver Enzyme Activities in Adult Male Rats.

		Saline group	Growth hormone group	t
Q_{urea}	Range	3.98–5.86 (9)	2.27–5.60 (9)	3.24*
	Mean	4.99	3.77	
	S.D.	.60	.96	
Arginase	Range	78–98 (9)	67–81 (9)	3.14*
	Mean	83	74	
	S.D.	7	5	
Aspartic-glutamic transaminase	Range	133–184 (8)	115–181 (9)	
	Mean	157	157	
	S.D.	16	23	
Alanine-glutamic transaminase	Range	96–146 (8)	70–124 (9)	2.83†
	Mean	120	101	
	S.D.	16	12	
Blood urea, mg %	Range	25.6–38.0 (9)	25.0–36.8 (8)	
	Mean	30.0	31.0	
	S.D.	3.9	3.9	

* Significant at the 1% level. † Significant at the 5% level.
( ) signifies No. of animals used.

weight, the average volume injected being 2.0 ml. The remaining group was similarly injected with saline (made slightly alkaline) on a body weight basis. Three hours following injections, the animals were sacrificed by stunning and decapitation. Blood was collected from each animal and heparinized for the determination of blood urea by the method of Archibald(6). Livers were quickly removed and aliquots taken for liver enzyme measurement. Liver urea formation was determined as the Q_{urea} of Krebs and Henseleit (7), defined as the number of μl of urea-CO_2 formed per mg dry tissue per hour. The procedure of Gornall and Hunter(8) was employed, modified in that the liver slices were agitated in Warburg flasks mounted on manometers instead of in small Erlenmeyer flasks. Liver arginase activity of homogenates was estimated by the method of Van Slyke and Archibald(9) as adapted to liver tissue by Liener and Schultze(10). Arginase activities were expressed as μmol urea formed per 100 mg wet tissue per hour. Activity of the aspartic-glutamic transaminase in liver homogenates was measured by the method of Tonhazy *et al.*(11). Activity of the alanine-glutamic transaminase in liver homogenates was estimated by substituting dl-alanine for dl-aspartate and omitting the aniline citrate step thus allowing direct measurement of the pyruvate formed(12). Transaminase activities are expressed as Q_T^{10}, defined as the μl pyruvate-CO_2 formed per mg wet tissue per hour. The results of these analyses are shown in Table I. A "t" test was used to ascertain statistical significance.

A further study was carried out in the same manner employing young male rats (107-135 g) with 5 animals in each group. The results of this study are shown in Table II and a "t" test was applied to ascertain statistical significance.

Discussion. The significant depression in liver arginase activity and in the rate of urea formation by liver slices, as a consequence of growth hormone administration, agree with a hypothesis that the hormone acts in part at least in suppressing amino acid catabolism. These findings offer direct evidence of the mechanism involved in the decreased appearance of blood urea following casein hydrolysate administration in growth hormone-treated, nephrectomized rats(2). The lowered alanine-glutamic transaminase activity observed in the growth hormone-treated rats suggests that this transaminase may be directly related to amino acid catabolism though further studies are necessary to verify such a hypothesis. Caldwell and McHenry(12) have reported that the aspartic-glutamic transaminase is less sensitive to basal diet changes than is the alanine-glutamic transaminase. This lowered sensitivity may explain the absence of an

TABLE II. Effects of Anterior Pituitary Growth Hormone on Liver Enzyme Activities in Young Male Rats.

		Saline group	Growth hormone group	t
Q_{urea}	Range	1.84–4.46 (5)	2.30–3.16 (5)	
	Mean	2.99	2.83	
	S.D.	1.04	.36	
Arginase	Range	64–80 (5)	65–76 (5)	
	Mean	71	71	
	S.D.	8	4	
Aspartic-glutamic transaminase	Range	120–158 (5)	113–162 (5)	
	Mean	138	131	
	S.D.	16	19	
Alanine-glutamic transaminase	Range	47–77 (5)	69–117 (5)	3.20*
	Mean	60	90	
	S.D.	14	20	
Blood urea, mg %	Range	22.0–36.4 (5)	18.2–32.6 (5)	
	Mean	27.2	25.2	
	S.D.	6.2	5.3	

* Significant at the 5% level.
( ) signifies No. of animals used.

effect of growth hormone on aspartic-glutamic transaminase.

Adult male rats were employed in the study reported and a second similar study, in which liver arginase activity was measured, confirmed the observation that growth hormone significantly depresses the liver arginase activity in adult male rats when determined on an individual basis. In a third study employing young male rats (107-135 g), treatment with growth hormone in the same manner as described caused no significant alteration in the rate of urea formation by liver slices, in aspartic-glutamic transaminase activity, or in liver arginase activity but caused a significant increase in alanine-glutamic transaminase activity. These findings support the view that effects of growth hormone are difficult to produce in the rapidly growing rat(13) and the effect of the hormone on liver alanine-glutamic transaminase may be dependent on the age of the animal. Comparison of values obtained for untreated young and adult animals reveals that the blood urea level and the liver enzyme activities measured were lower in young male rats than in adult male rats. This might be a consequence of the state of rapid growth, and hence higher rate of protein anabolism, of the young rats. The lack of response to growth hormone administration of liver enzyme activities in the young rats could conceivably be due to the fact that the livers of these animals were already responding maximally to a relative excess of endogenous growth hormone. In none of the experiments did growth hormone significantly alter the level of blood urea.

Summary. In the adult male rat, an acute dosage of anterior pituitary growth hormone caused significant depressions in the rate of urea formation by liver slices, in liver arginase activity, and in liver alanine-glutamic transaminase activity but did not alter liver aspartic-glutamic transaminase activity. None of these effects were observed in young male rats, and in such animals, a significant elevation in liver alanine-glutamic transaminase activity was noted.

This study was facilitated greatly by a Public Health Research Grant from the Department of National Health and Welfare. The supply of growth hormone was made available without charge by the National Research Council of Canada.

---

1. Hoberman, H. D., *Yale J. Biol. and Med.,* 1950, v22, 341.

2. Russell, J. A., *Endocrinol.,* 1951, v49, 99.

3. Friedberg, F., and Greenberg, D. M., *Arch. Biochem.,* 1948, v17, 193.

4. Bartlett, P. D., and Glynn, M., *J. Biol. Chem.,* 1950, v187, 261.

5. Fraenkel-Conrat, H., Simpson, M. E., and

Evans, H. M., *Am. J. Physiol.*, 1942, v138, 439.

6. Archibald, R. M., *J. Biol. Chem.*, 1945, v157, 507.
7. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, 1932, v210, 33.
8. Gornall, A. G., and Hunter, A., *J. Biol. Chem.*, 1943, v147, 593.
9. Van Slyke, D. D., and Archibald, R. M., *J. Biol. Chem.*, 1946, v165, 293.
10. Liener, I. E., and Schultze, M. O., *J. Biol. Chem.*, 1950, v187, 743.
11. Tonhazy, N. E., White, N. G., and Umbreit, W. W., *Arch. Biochem.*, 1950, v28, 36.
12. Caldwell, E. F., and McHenry, E. W., *Arch. Biochem. Biophys.*, 1953, in press.
13. Evans, H. M., and Simpson, M. E., *Am. J. Physiol.*, 1931, v98, 511.

Received June 29, 1953. P.S.E.B.M., 1953, v83.

Inactivation of Antidiuretic Hormone by Blood Serum of the Pregnant Woman.* (20490)

H. CROXATTO, C. VERA, AND L. BÁRNAFI.

From the Laboratorio de Fisiologia, Universidad Católica de Santiago, Chile.

It is assumed that the secretion of minimum amounts of antidiuretic hormone (vasopressin) by the hypophysis is sufficient to insure a normal reabsorption of water by the kidney. This amount corresponds to 1 to 5 mU per hour in the dog(1-3) and a similar amount in man(4,5). Furthermore, blood plasma and tissues contain enzymes that inactivate vasopressin and oxytocin(6-10). The oxytocin-inactivating ability increases considerably in the blood serum during pregnancy (11). An increase of 1000 times was observed by Page(12) from the time of conception to the ninth month of pregnancy. Similar observations were recorded by Croxatto(13) for the pressor effects of vasopressin.

No systematic study of the inactivation of the antidiuretic properties of vasopressin in pregnancy has been undertaken. Aside from the physiological importance of such study, it is of interest because of the difference in stability of the antidiuretic and pressor effects of vasopressin *in vitro*. The antidiuretic activity appears to be more resistant to changes in pH (Heller(14)). Ralli(15) using sodium thioglycollate, and Almeyda(16) and Croxatto(17) with thiosorbitol have shown that the pressor activity disappears completely or almost completely, whereas the antidiuretic activity is not affected. The present communication deals with the determination of the ability of blood serum from non-pregnant, pregnant and postpartum women to inactivate the antidiuretic effects of vasopressin.

Methods. Recently obtained blood serum, kept at a temperature of −5° was used throughout. A vasopressin preparation[†] containing 10 U per ml was added to the serum and the conveniently diluted mixture adjusted to pH 7.3 and incubated at 37°. The time of incubation varied from 30 to 300 minutes for the serum of pregnant women and from 1 to 24 hours for the non-pregnant. At the end of incubation, 1 ml of the solution per 100 g of body weight was injected intraperitoneally into 4 hyperhydrated rats following the technique of Burn(18). The inactivation of the antidiuretic hormone by the serum was measured by the effect on diuresis of the incubated material. Controls were run on the hormonal preparation and the sera *per se*. With injections of 0.1 to 0.2 ml of serum per 100 g body weight of the rat, the effect on diuresis was negligible and could be disregarded. A higher dose of serum produced antidiuretic effects (Croxatto and coworkers)(19,20). The same solutions assayed in rats were tested on the blood pressure of anesthetized cats, by injection into the femoral vein in small doses and at relatively long intervals, to determine whether or not the loss of pressor activity of the substrate paralleled the loss of antidiuretic activity.

---

* This work was supported by the Fundacion Gildemeister and the Rockefeller Foundation.

[†] Pituitrol Sanitas, Santiago (Chile).

The "antidiuretasic activity" of the sera of pregnant women at half and full term was determined by measuring the time required for the destruction of 50% of the antidiuretic potency of the added hormone. The "antidiuretasic units" were calculated applying the same formula ($\frac{0.693}{t}$ x 100 x dilution of the serum) proposed by Page(21) for the calculation of the "oxytocinasic units". The time (t) is that required for the destruction of 50% of the substrate and was determined by injecting, after different intervals of incubation, a mixture of serum and vasopressin into properly prepared rats.

The results obtained with blood sera of 50 pregnant women indicate that in the course of pregnancy there is a progressive increase in the enzymatic activity responsible for the destruction of the antidiuretic properties of vasopressin (Fig. 1). In the last week of pregnancy 0.1 ml of blood serum destroys 300 to 350 mU of vasopressin in the course of 40 to 50 minutes, whereas the same amount of blood serum of a nonpregnant woman inactivates only 30 to 35 mU in 960 minutes.

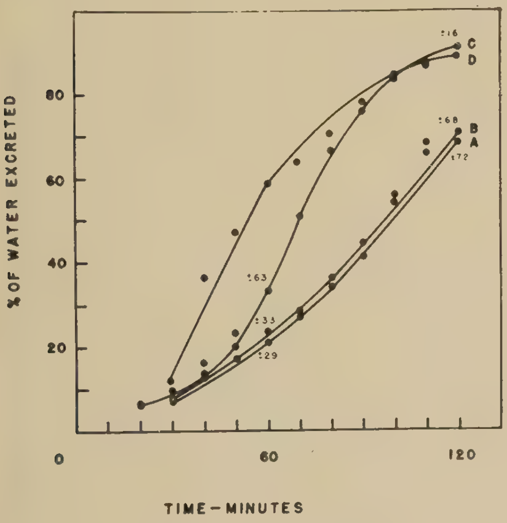


FIG. 1. Average percentages of urine volume excreted by 4 groups of 36 rats each, as related to amount of water administered by stomach tube (5% of body wt, Burn test) (18). Injected (intrap. per 100 g body wt) at: A. 1 ml of vasopressin solution containing 62.5 mU. B. 1 ml of vasopressin solution (62.5 mU) after 240 min. incubation with 0.01 ml of blood serum of non-pregnant woman. C. 1 ml of vasopressin solution (62.5 mU) after 240 min. incubation with 0.01 ml of blood serum from 9th month of pregnancy. D. 1 ml of 0.9% NaCl. Figures on the curves indicate stand. dev. of the averages (D.S.) at 60 and 120 min.

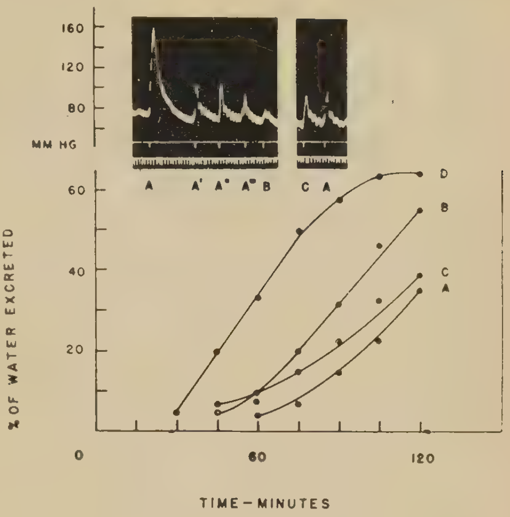


FIG. 2. Percentage of urine volume excreted by 4 groups, each of 4 rats, as related to amount of water administered by stomach tube (Burn test) (18). Intraperitoneal injection per 100 g of body wt at: A. 1 ml of vasopressin solution containing 62.5 mU. B. 1 ml of vasopressin solution (62.5 mU) after 60 min. incubation with 0.01 ml of blood serum (9th month of pregnancy). C. 1 ml same as B, incubated with 0.1 ml of blood serum of non-pregnant woman. D. 1 ml 0.9% NaCl. Upper part of the figure represents the variations of arterial pressure registered at the carotid of a cat under "Dial" anesthesia. Injected in the femoral vein with: A. 1 ml of vasopressin solution containing 125 mU. A.' 1 ml of vasopressin solution containing 90 mU; A." 1 ml of vasopressin solution containing 60 mU; A.‴ 1 ml of vasopressin solution containing 30 mU. B. 125 mU of vasopressin after 60 min. incubation with 0.02 ml of blood serum (9th month of pregnancy). C. 125 mU of vasopressin after 60 min. incubation with 0.02 ml of blood serum of non-pregnant woman.

The blood serum of women at half term showed from 16 to 20 U and that of women at term (9th month) 150 to 170 U of "antidiuretasic activity", calculated with the formula of Page. These values are similar to the ones obtained by Page, 15 U and 100 U respectively, for the "oxytocinasic activity".

The "antidiuretasic activity" rapidly decreases in the post-partum period and reaches the values preceding pregnancy 5 or 6 days after delivery.

The pressor activity, as determined by measuring arterial pressure in cats, (Fig. 2) is destroyed by blood sera at the same rate as the antidiuretic activity.

These observations lead to the assumption that the same enzyme is involved in the inactivation of both hormones, oxytocin and vasopressin. The large and progressive increase in the capacity to inactivate the antidiuretic property of the hormone during pregnancy, poses the question of why, as pregnancy progresses, there is no development of diabetes insipidus. This could be due to a large increase in the production of hormone by the hypophysis, thus compensating for the greater inactivating capacity of the blood serum or to the gradual development of another compensatory mechanism, as yet unknown, that would make unnecessary the action of the hormone. It is also possible that the fetus or the placenta produce large amounts of antidiuretic substances. The increase in "antidiuretasic activity" would be a regulatory mechanism to eliminate the excessive antidiuretic activity.

A more active autolysis of plasma proteins during pregnancy, would explain the larger liberation of antidiuretic substances similar to the hormone. It is known that hydrolysis of hypertensinogen and other proteins of plasma with pepsin produces considerable amounts of antidiuretic and oxytocic substances (Croxatto and coworkers(22)).

Summary. The "antidiuretasic activity" (activity responsible for the destruction of the antidiuretic properties of vasopressin) of the blood sera increases progressively in the course of pregnancy. In the last week of pregnancy 0.1 ml of blood serum destroys 300 to 350 mU of vasopressin in 40 to 50 minutes, whereas in the nonpregnant woman it inactivates only 30 to 35 mU in 960 minutes. The "antidiuretasic activity" rapidly decreases in the post-partum period and reaches the values preceding pregnancy after 5 or 6 days of delivery.

---

1. Shannon, J. A., *J. Exp. Med.*, 1942, v76, 387.
2. O'Connor, W. J., and Verney, E. B., *Quart. J. Exp. Physiol.*, 1942, v31, 393.
3. Verney, E. B., *Lancet*, 1946, v739, 781.
4. Lauson, A., Eder, H. A., Chinard, F., Cotzias, G. C., and Greif, R. L., *Fed. Proc.*, 1948, v7, 69.
5. Lauson, H. D., *Am. J. Med.*, 1951, v11, 135.
6. Heller, H., and Urban, F. F., *J. Physiol.*, 1935, v85, 502.
7. Jones, A. M., and Schlapp, W., *J. Physiol.*, 1936, v87, 144.
8. Larson, E., *J. Pharmacol.*, 1938, v62, 346.
9. Croxatto, H., Croxatto, R., and Alliende, J., *Rev. de la Soc. argent. de Biol.*, 1942, v18, 441.
10. Birnie, J. N., Jenkins, R., Boss, W. R., Osborn, C. M., and Gaunt, R., *Fed. Proc.*, 1949, v8, 12.
11. Von Fekete, K., *Endokrinologie*, 1930, v7, 364.
12. Page, E. W., *Am. J. Obst. and Gyn.*, 1946, v52, 6.
13. Croxatto, H., Reyes, M., and Croxatto, R., *J. chilenas Obst. Ginec.*, 1947, v1, 469.
14. Heller, H., *J. Physiol.*, 1939, v96, 337.
15. Ralli, E. P., Raisz, L. G., Leslie, S. H., Dumm, M. E., and Laken, B., *Am. J. Physiol.*, 1950, v163, 141.
16. Almeyda, L., Tesis de Grado, *Univ. de Chile*, 1951.
17. Croxatto, H., Almeyda, L., and Barnafi, L., in press, 1953.
18. Burn, H. J., *Quart. J. Pharm.*, 1931, v4, 517.
19. Croxatto, H., Andrade, F., and Bárnafi, L., *Acta Physiol. Lat. Am.*, 1952, v2, 142.
20. Croxatto, H., Bárnafi, L., and Ferrer, R., in press, 1953a.
21. Page, W. E., *Science*, 1947, v105, 292.
22. Croxatto, H., Rojas, G., and Bárnafi, L., *Acta Physiol. Lat. Am.*, 1951, v1, 178.

Received June 29, 1953. P.S.E.B.M., 1953, v83.

Inhibition of Cytopathogenic Effect of Poliomyelitis Viruses in Tissue Culture by Antibiotic M-8450. (20491)

Robert N. Hull and Janet M. Lavelle. (Introduced by H. M. Powell.)
From the Lilly Research Laboratories, Indianapolis, Ind.

The chemoprophylactic effect of a mold filtrate designated M-8450 against MM and Semliki Forest viruses in white mice has been described by Powell *et al.*(1). Parenteral administration of M-8450, 24 hours prior to infection protected the mice against approximately 100 LD_{50} of MM virus. Shope(2) has reported similar data obtained from mice infected with Columbia SK encephalomyelitis virus treated with a mold preparation indentified as helenine. Powell and Culbertson(3) reported the inhibition in mice of a type II poliomyelitis virus, MEF1, by antibiotic M-8450.

Since types I and III poliomyelitis viruses do not readily infect mice or other small laboratory animals, an evaluation of M-8450 against these types had to made in other ways. Tissue culture appeared to be one possibility. Demonstration of protection in monkeys, however, would be most desirable. The following is a report of the methods and results of preliminary studies of the inhibitory action of this mold filtrate on examples of the 3 immunological types of poliomyelitis virus grown in tissue culture.

Materials. Tissue cultures. Plasma clot roller tube cultures of the type described previously(4) were employed with testicular tissue from immature cynomolgus or rhesus monkeys. Approximately 6 explants/tube were used and were fed a medium of 30% horse serum (HS), 20% chick embryo extract (EE_{50}), and 50% Earle's salt solution (BSS). 100 units each of penicillin and streptomycin per ml were included in this medium. The cultures were incubated at 37.5°C for 7 to 14 days until sufficient outgrowth had occurred to make them satisfactory for virus growth. At the time of virus inoculation the medium was altered to one of 30% HS, 30% serum ultrafiltrate (OX) (SUF), 5% EE_{50}, or an ultrafiltrate of EE_{50}, and 35% BSS.

Viruses. Two Type 1 viruses labeled Duffy and 787, and a type 3, Leon, were very kindly supplied to us by Dr. John F. Enders, The Children's Hospital, Boston. A cytopathogenic Type 2 strain, YSK, was obtained through the courtesy of Dr. Jerome T. Syverton, University of Minnesota. These viruses have been maintained in our laboratory through tissue culture passages and occasionally retyped with specific antisera to check their immunological type identity. The inocula in all experiments referred to in this paper have been 0.1 ml of the undiluted tissue culture fluid of a previous virus passage. Periodic titrations of these fluids have shown the virus titers to be of the order of 10^{-5} to 10^{-6+} ID_{50}'s/0.1 ml of the fluid.

Antibiotic M-8450. This material was in the form of a crude broth which had been harvested aseptically and to which no preservative had been added. No toxicity of the antibiotic was evident at the levels studied. The activity of each lot employed was determined by assay in mice against MM virus.

Methods. The procedure generally followed was as follows: the old culture medium is removed, the M-8450 is applied directly to the cells for a 10 minute period, 1.0 ml of new medium (on occasion the old medium was returned) is added, the cultures are incubated for 24 hours, the medium is again withdrawn, virus inoculation is made directly onto the cells, and the medium is replaced. The M-8450 was also added just 10 minutes before virus and simultaneously with it in some experiments. The dose of M-8450 in these experiments was 0.1 ml of a 1:10 dilution. Another technic of treatment also employed consisted of removing the old medium, saturating the cells with 1.0 ml of the M-8450, incubating for 4 hours on the roller tube apparatus, removing the M-8450, inoculating virus and adding new medium.

Results. In these preliminary tissue culture studies of the antiviral activity of antibiotic M-8450, it has been demonstrated that this

TABLE I. Effect of M-8450 on Virus Growth.

Culture No.	Virus type	Treatment	Results, days after virus inoculation							Results of 5th day subcultures, days
			4th	5th*†	6th	7th	8th*	11th	12th	
T-42	I	Virus controls	++++	‡						++++ 3
T-43	787		++++	‡						
T-46		24 hr pre-treated	0	0	0	0	0	0	0	++++ 6
T-47		with 8450	0	0 lost	—	—	—	—	—	
T-49			0	0	0	0	0	0	0	
T-16	II	Virus controls	+	+++	+++					++++ 5
T-17	YSK		+	+++	+++					
T-26		24 hr pre-treated	0	0	0	0	0	0	0	0 14
T-29		with 8450	0	0	0	0	0	0	0	
T-33			0	0	0	0	0	0	0	
T-34	III	Virus controls	++++	‡						++++ 4
T-36	Leon		+++	‡						
T-37		24 hr pre-treated	0	0	0	0	0	0	0	++++ 7
T-40		with 8450	0	0	0	0	0	0	0	
T-41			0	0	0	0	0	0	0	

* Culture medium changed, 10-fold dilution made of original drug and virus.

† All 6 groups subcultured.

‡ Discarded.

++++ = complete cell damage; 0 = no damage.

mold filtrate will protect monkey testicular cells growing in tissue culture from the destructive action of all of the samples of the 3 immunological types of poliomyelitis viruses employed. Like the previous reports by Powell *et al.*, the M-8450 again was found to be most active when the cells were treated with it before the introduction of virus. Incubation of mixtures of M-8450 and virus for 24 hours prior to inoculating cultures caused no inhibition of viral activity in the culture. One-tenth ml of a 1:10 dilution of M-8450 placed on the cells, as described above, 24 hours before inoculation with approximately 100,000 ID_{50} (titered) of a Type 1 virus (Duffy) almost completely protected the cells against virus damage. The untreated controls were completely destroyed after 3 days incubation. When the M-8450 was administered just 10 minutes before virus inoculation, or simultaneously with it, no protection was afforded. Similar observations were made employing Type 2 (YSK), and Type 3 (Leon) viruses, with the exception that complete protection was obtained against these viruses following 24 hour pretreatment. Repetitions of the experiment with Type 1 virus have also demonstrated complete protection of the cells against this type. Pretreatment of cells with the uninoculated mold culture broth produces no such antiviral effect.

The second method of study mentioned above involving saturation of the cells with 1.0 ml of the M-8450 was also found to afford protection against a Type 1 virus (787) in some experiments. This treatment rendered the cells completely refractory to the virus while untreated controls were completely destroyed in 3 days. A similar experiment done at another time employing the Type 3 (Leon) virus failed to demonstrate protection of the cells. This finding with the Leon virus has not been reexamined nor explained at this time, but further testing is planned.

In more recent studies the protective effect of the antibiotic has been demonstrated upon addition of 0.1 ml of a 1:10 dilution to the tissue culture medium 24 hours before virus inoculation. Thus the 10 minute period during which the cells were directly exposed to the M-8450 alone before culture medium was added has been eliminated. Further study of the value of this 10 minute period of direct treatment to the cells has revealed that removing the M-8450 and rinsing the cells with BSS before addition of medium, and 24 hours incubation prior to virus inoculation, does not leave the cells protected against virus damage. The data reported in this paper, however, were obtained from studies in which the 10 minute period of direct treatment had been employed without subsequent removal of the M-8450 or rinsing of the cells.

Table I illustrates the data obtained from a typical experiment employing all 3 types of virus and the 24 hour pretreatment schedule of M-8450. As can be seen, the 787 and Leon virus controls were destroyed in 4 days, while the M-8450 treated cultures inoculated with these viruses revealed no evidence of virus destruction during the 12 day observation period. Destruction of cells by YSK virus was slower than desired, indicating less virus to be present than in the 787 and Leon controls. The M-8450 treated cultures challenged with the YSK virus again failed to exhibit virus cytopathology throughout the course of the experiment. The subcultures from each of the virus controls were destroyed in the usual time proving the presence of virus in these damaged cultures. The subcultures from the treated cultures in the 787 and Leon series also were destroyed, but the time required was almost twice as long as with their respective controls. The virus demonstrated to be present in the treated and undamaged cultures probably represented a part of the inoculum, as similar observations have been noted when attempts were made to culture poliomyelitis viruses in cell types not suitable for their growth, *e.g.*, chick embryo cells. The failure to recover the YSK virus from the treated cultures in this experiment may have been due to a smaller content of virus in the original inoculum. As mentioned in the section on materials, our virus stocks have frequently been rechecked for their immunological type specificity. Such examinations made with specific antisera before and after these studies have indicated that each strain of virus has maintained its type specificity as labeled in Table I. The observation that YSK infected mice produced typical symptoms provided additional evidence of the identity of this virus, whereas 787, Duffy and Leon viruses failed to infect the mice. The Duffy has produced paralysis in monkeys within 4 days. Neither the 787 nor Leon viruses have been studied in monkeys.

Discussion. The results obtained in these studies show quite clearly that exposure of monkey testicular cells in tissue culture to relatively small amounts of antibiotic M-8450, 24 hours before inoculation with any of the samples of the 3 immunological types of poliomyelitis viruses causes the cells to become refractory to virus infection. This finding agrees very well with the previous observations of *in vivo* tests in mice against various neurotropic viruses. That the antibiotic exerts its effect on the cells, rather than the virus, is indicated both by the fact that pretreatment of the cells is necessary to demonstrate the protection whereas pretreatment of the virus has no such effect, and by the observation that viable virus can be demonstrated in the undamaged, treated cultures as long as 5 days after inoculation. In the mouse, efforts to recover virus (MM) from treated animals have failed, but it is likely that the intact animal is capable of eliminating the non-infecting virus by several routes. In the culture these mechanisms for elimination are not present, and since poliomyelitis viruses are stable, the inoculum will survive even though no reproduction occurs. Thus the M-8450 appears to have no neutralizing effect on the virus itself under the conditions studied in tissue cultures.

The explanation of the mechanism of the action of antibiotic M-8450 is an intriguing question and one which is under consideration. Evidence now available points to the probability that the mode of action may be one of interrupting or preventing some stage of intracellular multiplication of virus. An effect on virus attachment to the cell is not precluded; however, the need for a period of pretreatment decreases the attractiveness of such an explanation. Study of the mechanism of action may lead to an insight into the processes occurring during virus reproduction. Such information might in the long run be of more value to the whole problem of poliomyelitis infection than any immediate significance of antibiotic M-8450 as a possible treatment for this disease.

Summary. Pretreatment of tissue cultured monkey testicular cells with antibiotic M-8450 inhibits the cytopathogenic effect of all 3 immunological types of poliomyelitis virus

tested, but does not destroy or neutralize the viruses.

---

1. Powell, H. M., *et al.*, *Antibiotic and Chemother.*, 1952, v2, 432.

2. Shope, R. E., *J. Exp. Med.*, 1953, v95, 601.

3. Powell, H. M., and Culbertson, C. G., Proc. Soc. Exp. Biol. and Med., 1953, v83, 161.

4. Hull, R. N., *Science*, 1953, v117, 223.

Received July 1, 1953. P.S.E.B.M., 1953, v83.

Effect of Adrenocorticotropic Hormone on Pneumococcal Infections in Rabbits. (20492)

Harry J. Robinson, Richard C. Mason, David H. Johnson, and Amelia L. Smith.
(Introduced by Hans Molitor.)

From the Merck Institute for Therapeutic Research, Rahway, N. J.

Many investigators have reported on the effects of large doses of cortisone and Compound F on the resistance of various animal species to a variety of infections(1-20). Shwartzman(21) has shown that adrenocorticotropic hormone (ACTH) and cortisone in combination, or cortisone alone, produced a marked acceleration of poliomyelitis infection in mice and an enhancement in the susceptibility of hamsters to this infection. He has reported, however, that ACTH alone failed to produce this effect and hence has postulated that the adrenals may elaborate an unknown factor capable of reversing the enhancing effects of cortisone on infection. It seemed important, in view of the possible widespread significance of these observations, to determine if Shwartzman's findings applied to other infections and to other species. The present report is concerned with the effect of ACTH on an experimental pneumococcal infection in rabbits.

Methods. Male albino rabbits, weighing between 2.0 and 2.5 kg each, were infected intradermally with a Type I pneumococcus by injecting into the shaved abdominal skin 0.1 cc of a 2 x 10^{-3} dilution of a 6-hour culture grown in nutrient broth containing 5% defibrinated rabbit blood. The virulence of the culture was such that 0.5 cc of a 10^{-9} dilution (1-3 organisms) was lethal for a mouse. The size of the skin lesion resulting from the injection of the pneumococci was measured by tracing the periphery of the lesion on transparent paper and the area of the skin lesion was subsequently obtained by retracing the paper outline with a planimeter. The blood culture studies were performed by plating 0.1 cc of blood obtained from the marginal ear vein in 5% blood agar. Serial 10-fold dilutions of the blood were plated in order to assure accuracy in determining the number of bacterial cells. The plates were then incubated for 24 hours at 37°C.

A sample of crude ACTH, equivalent to Armour Standard La-1-A, was given in doses of 0.25 mg to 2.0 mg every hour night and day. Treatment was initiated 18 hours before the bacterial injection and then continued for 96 hours or until death of the animal. Similar studies were repeated with a highly purified ACTH containing 200-300 units per mg (22) with essentially the same results.

Results. Survival studies. The data presented in Table I indicate that 76% of the control animals survived the infection. Animals treated with ACTH, in contrast, began to die within 24-48 hours after the bacterial injection, and only 17% of the animals receiving ACTH survived the 10-day observation period. These data indicate that ACTH is capable of decreasing the survival of rabbits

TABLE I. Survival of Animals Treated with ACTH and Infected Intradermally with *D. pneumoniae.*

		Dose ACTH in mg/hr			
	Saline	.25	.5	1.0	2.0
No. of animals	17	6	6	17	6
% survival	76	17	17	18	17

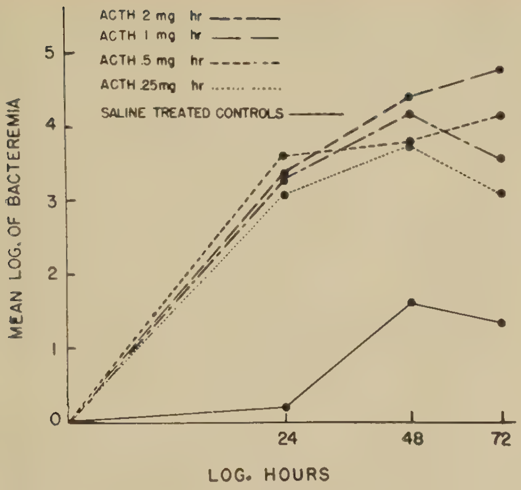


FIG. 1. Bacteremia in rabbits treated with ACTH.

infected with a Type I pneumococcus. Control studies performed with the same doses of ACTH in non-infected animals showed that these quantities of the hormone were not lethal. Animals that received ACTH, however, developed a marked lipemia and in some cases the total lipid content of the serum reached a value of 5 g %. Adrenal hypertrophy was observed in the animals which received ACTH.

Blood culture studies. The control rabbits developed a transient bacteremia which had its onset approximately 4 hours after the intradermal injection of the pneumococci and reached a peak at 48 hours after the injection (Fig. 1). The bacteremia then gradually subsided and the blood was sterile in most cases at the 120-hour observation period. The animals receiving ACTH, in contrast, developed an early onset of sepsis and within 24-48 hours large numbers of pneumococci were found in the blood. Overwhelming sepsis occurred, in most cases, within 72 hours. Occasionally, animals receiving ACTH died 6 hours after the bacterial injection and numerous pneumococci were found in the blood.

Skin lesions. There were marked differences in the size and character of the skin lesion in the control and ACTH treated animals (Fig. 2). In the control animals the lesion reached a maximal size of approximately 50 sq cm within 48 hours. It remained essentially the same size for the next 4 or 5 days and then gradually subsided over the ensuing 15-day period. The lesions in the animals receiving ACTH developed very slowly and appeared as small flat areas with little evidence of inflammation.

The microscopic picture of the skin lesions in the 2 groups of animals offered a striking contrast. In the control animals, there was an infiltration of polymorphonuclear leucocytes at the bacterial injection site within a few minutes after infection was initiated, and these cells accumulated rapidly within the next few hours. Relatively few pneumococci were observed and they were found mainly within the leucocytes in the saline treated controls.

In the ACTH treated rabbits the inflammatory reaction was markedly suppressed and relatively few polymorphonuclear leucocytes, and little edema fluid, were found even 18 hours after the bacterial injection. Numerous pneumococci were observed in the tissue and blood vessels surrounding the injection site and animals frequently died of overwhelming infection with little evidence of local inflammation.

Discussion. Under the conditions of this study, rabbits receiving ACTH show a low-

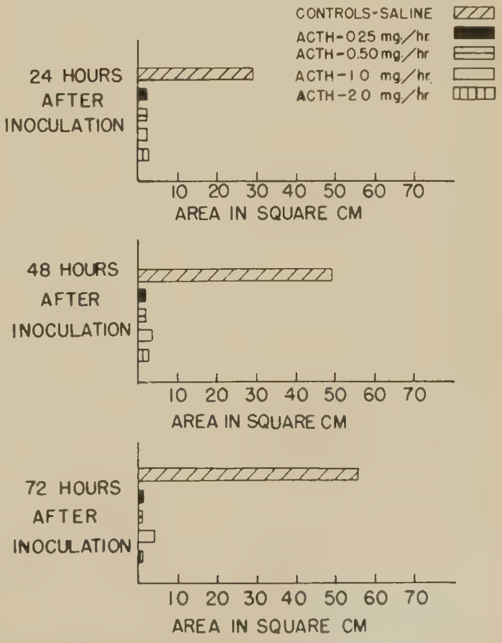


FIG. 2. Skin lesion size in rabbits treated with ACTH.

ered resistance to an experimental pneumococcal infection. The basic response of the animals receiving ACTH was essentially the same as that observed in rabbits treated with cortisone and Compound F(23). There was a marked suppression in both the cortisone and ACTH treated animals in the rate of mobilization of polymorphonuclear leucocytes at the injection site. The suppression of the inflammatory response, however, was greater and persisted for a longer period of time in animals receiving ACTH(23). Also there was essentially complete suppression of edema fluid formation under the influence of ACTH. It is not possible to state at this time whether these differences in response were related to variations in dosage and rate of absorption of the 2 materials, or to an actual difference in the effects of the hormones.

The data presented in this study do not support the hypothesis that ACTH in the rabbit can cause the secretion of a factor that counteracts the adverse effects of cortisone on pneumococcal infections, as Shwartzman has suggested for poliomyelitis in mice and hamsters(21). It is recognized that the experimental procedure used in the present study differed from that employed by Shwartzman, particularly with regard to the infectious agent and dosage schedules. Shwartzman's studies employed poliomyelitis while the studies reported here used a Type I pneumococcal infection. The ACTH was injected either once or 3 times a day in the studies reported by Shwartzman, and every hour, night and day, in our studies. This dosing schedule was undertaken in view of the findings of Ingle (24) that the effects of ACTH could best be demonstrated by maintaining constant tissue and blood levels. The adrenal cortices, in the present studies, were markedly hypertrophied and the hyperglycemia, lipemia, and atrophy of lymphoid tissue indicated a pronounced hyperadrenocorticism.

Summary. ACTH, given at frequent intervals and in large doses, lowers the resistance of rabbits to a Type I pneumococcal infection. ACTH appears to suppress inflammation at the bacterial injection site sufficiently to permit early and rapid invasion of the blood stream and surrounding tissues.

---

1. Mogabgab, W. J., and Thomas, L., Meeting of Central Soc. for Clin. Research, Nov. 3-4, 1950, Chicago.

2. ———, *J. Lab. and Clin. Med.*, 1950, v36, 968.

3. Kass, E. H., and Finland, M., *New England J. Med.*, 1951, v244, 464.

4. White, R. G., and Marshall, A. H. E., *Lancet*, 1951, v1, 891.

5. Robinson, H. J., *Fed. Proc.*, 1951, v10, 332.

6. ———, *The Suprarenal Cortex,* Proc. of Colston Research Soc. 5th Symposium, 1952, p. 105, London, Butterworths.

7. D'Arcy Hart, P., and Rees, R. J. W., *Lancet*, 1950, v2, 391.

8. Michael, M., Jr., Cummings, M. M., and Bloom, W. L., Proc. Soc. Exp. Biol. and Med., 1950, v75, 613.

9. Spain, D. M., and Molomut, N., *Am. Rev. Tuberc.*, 1950, v62, 337.

10. Spain, D. M., Abstr. 47th Ann. Meet., Nat. Tuberc. Assn., p. 4, May 14-18, 1951.

11. LaMaistre, C., and Tompsett, R., *Am. Rev. Tuberc.*, 1951, v64, 295.

12. Turner, T. B., and Hollander, D. H., *Bull. Johns Hopkins Hosp.*, 1950, v87, 505.

13. Cavallero, C., and Sala, G., *Lancet*, 1951, v175, (letter to the editor).

14. Abernathy, R., *J. Clin. Invest.*, 1951, v30, 626.

15. Vollmer, E. P., and Hurlbut, H. S., *J. Infect. Dis.*, 1951, v89, 103.

16. Shwartzman, G., *Am. J. Path.*, 1951, v27, 714.

17. Kass, E. H., Ingbar, S. H., Lundgren, M. M., and Finland, M., *J. Lab. and Clin. Med.*, 1951, v37, 780.

18. Kligman, A. M., Baldridge, G. D., Rebell, G., and Pillsbury, D. M., *J. Lab. and Clin. Med.*, 1951, v37, 615.

19. Wolf, A., Kabat, E. A., Bezer, A. E., and Fonseca, J. R. C., *Fed. Proc.*, 1951, v10, 375.

20. Schmidt, L. H., and Squires, W. L., *J. Exp. Med.*, 1951, v94, 501.

21. Shwartzman, G., Proc. Soc. Exp. Biol. and Med., 1950, v75, 835.

22. Brink, N. G., Kuehl, F. A., Jr., Richter, J. W., Bazemore, A. W., Meisinger, M. A. P., Ayer, D. E., and Folkers, K., *J.A.C.S.*, 1952, v74, 2120.

23. Robinson, H. J., and Smith, A. L., *Ann. N. Y. Acad. Sci.*, 1953, v56, 757.

24. Ingle, D. J., *Pituitary-Adrenal Function,* AAAS Symposium, 1949.

Received July 3, 1953. P.S.E.B.M., 1953, v83.

Study of Nine Rabies Street Virus Strains in the Syrian Hamster. (20493)

Reginald L. Reagan, Mildred T. Stewart, and Arthur L. Brueckner.

From the Virus Laboratory, Live Stock Sanitary Service, University of Maryland, College Park, Md.

Specific inclusion bodies present in the central nervous systems of animals dying from rabies are called "Negri Bodies"(1,2). These are most frequently demonstrated within, but are revealed occasionally outside the nerve cells. A definite diagnosis of rabies is made upon demonstration of these bodies in stained smears. These inclusions are eosinophilic bodies, usually spherical, but may be of other shapes, and vary from 1 to 30 μ in size. The virus structure shows basophilic granules. At present the animal most frequently used for the diagnosis of rabies is the mouse. A positive diagnosis is made on the development of symptoms of rabies in the mouse and the presence of Negri bodies in the mouse brain. However, the Negri bodies are frequently small and few in number, which sometimes makes their detection difficult. In previous studies the authors found the Negri bodies present in the brains of rabies-infected hamsters to be large and quite numerous, and the authors found in one instance, that a rabies specimen was so grossly contaminated with bacteria that all hamsters and mice inoculated intracerebrally succumbed to bacterial infection within the first 24 hours, whereas those hamsters challenged rectally were unaffected by the contaminants and came down several days later with rabies symptoms, and the brains of these hamsters contained numerous "Negri bodies"(3). The present study was undertaken to compare contaminated dog brains and brain material not contaminated from the same brain specimen and the effect in hamsters after instillation of these specimens rectally.

Materials and methods. The strains of rabies virus used in this study were obtained from Dr. I. M. Moulthrop of the Live Stock Sanitary Service Branch Laboratory of Salisbury, Md. These strains are as follows: 25077 (dog brain), 25148 (dog brain), 25122 (dog brain), 25256 (dog brain), 25116 (dog brain), 25140 (dog brain), 25115 (dog brain), 25108 (dog brain), 25025 (dog brain).

These specimens had been proven positive for rabies at Dr. Moulthrop's laboratory. One half of each brain specimen was preserved in glycerin and stored in a –4°C ice box and the other half was placed in a separate jar and frozen in a –40°C deep freeze storage vault until initiation of the present study. The specimens were removed from the –40°C deep freeze and left at room temperature for several days until they ground in mortars with alundum and diluted to 20% suspensions with physiological saline. Each suspension was cultured in thioglycollate broth, and all were found to be contaminated with bacteria. The other half of each brain specimen was removed from the glycerin, ground in mortars with alundum, and also diluted to 20% suspensions with physiological saline. Each of these specimens was cultured in thioglycollate broth, and all were free from bacteria. Diagnosis of rabies in hamsters and mice was made on the following factors: (1) virus symptoms present in hamsters and mice and (2) demonstration of Negri bodies in the Ammon's horn of the hamster and mouse brain. The presence of Negri bodies was determined by staining touch preparations of the Ammon's horn with Sellers stain(4) and (3) examining the slides under an optical microscope.

Seventy-two healthy female Swiss albino mice, age 21 days, were divided into 18 groups of 4 animals each. Each group from No. 1 to No. 9 was inoculated intracerebrally with one of the contaminated rabies-bearing brain suspensions, and Group No. 10 to Group No. 18 was inoculated intracerebrally with the other half of each rabies-bearing brain suspension free of bacteria. Each mouse received 0.03 cc. Seventy-two healthy Syrian hamsters, age 21 days, were divided into 18 groups of 4 animals each. Into each group from No. 1 to No. 9 was administered one of the above contaminated virus bearing dog brain suspensions by rectal instillation, and

TABLE I. Response of Hamsters to Rabies Street Virus Administered Rectally from Contaminated Bacterial Suspensions and Bacteria Free Suspensions. Four hamsters exposed in each series. Negri bodies numerous in each series.

Group No.	Virus strain	Min to max incubation period, days	Type of rabies	
			Dumb	Furious
	Brain contaminated with bacteria			
1	25077			
2	25148	6-8	4	
3	25122	6-7	4	
4	25256	8-16	4	
5	25116			
6	25140	8-9		4
7	25115	14-16	4	
8	25108			
9	25025	14-15	3	
	Bacteria free			
10	25077	6-8	4	
11	25148	7-10	4	
12	25122	14-16	3	
13	25256	14-16	3	
14	25116	6-8	4	
15	25140	5-6		4
16	25115	5-6		4
17	25108	5-6		4
18	25025	14-16	3	

into each group from No. 10 to No. 18 was administered one of the above virus bearing dog brain suspensions free of bacteria by rectal instillation. Each hamster received 0.1 cc. For the rectal instillation, the end of an 18-gauge needle was filed off, rough ends smoothed down, and the tip lubricated with vaseline before being inserted.

Results. All Swiss albino mice inoculated intracerebrally with the bacteria contaminated specimens succumbed to bacterial infection within 24 hours. All Swiss albino mice inoculated intracerebrally with the bacteria-free suspensions showed symptoms of rabies between 6 to 8 days. The animals were sacrificed when symptoms of rabies appeared. By using the Seller stain technic numerous Negri bodies were found to be present in the brains of the infected animals.

Fifty-six hamsters which had received the virus bearing brain suspension rectally from the contaminated and bacteria free dog brain suspensions showed symptoms of rabies between the 6th and 16th days. The inoculation periods and the form of rabies (furious or dumb) for each strain from both groups are given in Table I. The animals were sacrificed when symptoms of rabies appeared. By using the Sellers stain technic numerous Negri bodies were found to be present in the brains of the infected animals. The hamsters that showed no rabies symptoms were discarded at the end of a 30 day observation period.

Summary. Nine dog brains found positive for rabies were divided into 18 groups. One half of each dog brain was preserved in glycerin, and the other half was contaminated with bacteria. The infected dog brains were instilled rectally in hamsters. All glycerinated specimens produced rabies in hamsters while only 65 of the contaminated specimens produced rabies in hamsters. The glycerinated specimens injected intracerebrally in mice produced rabies in all mice between 6 to 8 days while all mice inoculated with the contaminated specimens succumbed within 24 hours.

---

1. Negri, A., *Z. F. Hyg. u Infektionskr.*, 1903, v43, 507.

2. ———, *Z. F. Hyg. u Infectionskr.*, 1903, v44, 519.

3. Reagan, Reginald L., Day, William C., and Brueckner, A. L., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 654.

4. Sellers, T. F., *Am. J. Pub. Health*, 1927, v17, 1080.

Received July 8, 1953. P.S.E.B.M., 1953, v83.

Electrolyte and Water Responses of Normal Subjects During Cortisone Administration.* (20494)

HENRY J. KOWALSKI, WILLIAM E. REYNOLDS, AND DAVID D. RUTSTEIN.
(With the technical assistance of Mrs. Ruth I. Harris, Mrs. Joan Shoulberg, and
Miss Mary Kascht.)
(Introduced by Maxwell Finland.)
From the Department of Preventive Medicine, Harvard Medical School

During studies in which cortisone acetate was administered to healthy young subjects, estimations of Evans blue space, thiocyanate space, serum electrolyte concentration and urine electrolyte excretion were made. Such information, generally not available from studies of normal subjects, is here reported.

Methods. Five ambulatory male medical students ranging in age from 22-26 years were studied during the intramuscular administration of cortisone acetate in doses of 50 mg 4 times per day for periods of 3 to 5 days. One subject was also observed while receiving cortisone acetate orally in doses of 50 mg 4 times per day for 4 days.

The meals of each subject were supervised by a medical dietitian[†] who selected a fixed diet resembling that ordinarily consumed by the individual. The caloric content of these diets ranged from 2400-3200 calories. The intake of sodium chloride was fixed at the usual daily level for each subject and ranged from 9-12 g daily. Water was consumed *ad libitum.*

Experiments were divided into 3 periods: Pretreatment, 4 or more days; treatment, 3 to 5 days; and post-treatment, 5 or more days.

All observations were made daily while the subjects were fasting and recumbent.

Blood in double oxalate(1) collected daily at 8:00 A.M. was used for the following determinations in duplicate: Hematocrit (3,000 r.p.m. for 30 minutes); red blood cell count, using 2 certified pipettes (an average of 4 counts, 2 from each pipette); and hemoglobin by the alkaline hematin method using the Evelyn macrocolorimeter(2). Serum was analyzed for sodium and potassium using a flame photometer with an internal lithium standard(3) and for chloride(4).

The freezing point depression(5,6) was determined by means of an electrical resistance thermometer (Western Electric Co., Thermistor No. 14A). The reproducibility of this method is demonstrated by the fact that 15 determinations on a standard glucose solution, each performed on different days, showed a mean freezing point of —0.520°C with a standard error of ±0.001°C.

A daily 24-hour urine collection was analyzed for sodium(3), potassium(3), and chloride(4).

Evans blue space was determined by a modification(7) of the method of Gibson and Evans(8) using specimens of serum from venous blood collected at 15, 30, 45, and 60 minutes after the injection of 10 ml of a 0.1% solution of the dye. Thiocyanate space was determined by a modification(7) of the method of Crandall and Anderson(9) using serum from blood collected at one, 2, and 3 hours after the intravenous injection of 8 ml of a 10% solution of thiocyanate. Thiocyanate space was not corrected for binding by albumin(10) since the correction for albumin in extra-vascular fluid is not feasible(11).

Result. A. *Blood and serum studies.* (Table I). (Values in Table I are averages of individual means which were computed for each subject by period.) Erythrocyte, hemoglobin, and hematocrit values decreased slightly during drug administration, and after treatment they approached but did not reach pretreatment figures. Serum electrolyte concentration and freezing point depression were unaltered during drug administration and in the post-treatment period.

---

* Supported in part by grants from the National Heart Institute (Research Grant H-177-C2) and from the Lederle Laboratories Division, American Cyanamid Co.

[†] We are indebted to Dr. George W. Thorn for this service.

TABLE I. Mean Erythrocyte, Hemoglobin, Hematocrit, Serum Electrolyte and Serum Freezing Point during Experiments with Cortisone Acetate. Mean ± standard deviation of sample.

	Pre-treatment period	Treatment period	Post-treatment period
R.B.C., cells/mm^3	4.95 ± .56*	4.47 ± .46	4.62 ± .20
Hgb, g/100 cc	15.1 ± 1.5 * (14.8)†	14.2 ± 1.5 (13.2)†	14.4 ± 1.0 (13.4)†
Hematocrit, %	47.5 ± 4.7 * (43)	42.5 ± 2.6 (39)	43.2 ± 2.9 (41)
Serum Na$^+$, mEq/L	142.2 ± 3.4 * (142)	139.6 ± 1.5 (145)	139.0 ± 3.4 (148)
Serum K$^+$, mEq/L	4.2 ± .6 * (4.2)	4.0 ± .2 (3.9)	4.1 ± .2 (3.7)
Serum Cl$^-$, mEq/L	107.6 ± 1.14* (104.3)	107.2 ± 2.16 (105.5)	107.6 ± 1.14 (103.8)
Serum freezing point, °C	−.546 ± .008*	−.547 ± .013	−.546 ± .011

* Mean ± stand. dev. during intramuscular administration.
† Figures in parentheses are the values of subject 1 during oral administration.

TABLE II. Urine Volume and Electrolyte Excretion during Experiments with Intramuscular Cortisone Acetate.

	Subject	Pre-treatment period	Treatment period	Post-treatment period
Na$^+$, mEq/24 hr	1	155 (132)*	103 (109)*	201 (221)*
	2	150	153	194
	3	158	104	252
	4	130	90	212
	5	151	75	140
	Mean	149	105	200
K$^+$, mEq/24 hr	1	71 (81)	121 (82)	73 (90)
	2	102	94	75
	3	74	71	68
	4	89	96	68
	5	71	121	73
	Mean	86	90	71
Cl$^-$, mEq/24 hr	1	140 (147)	130 (133)	207 (221)
	2	188	170	180
	3	216	158	230
	4	124	110	227
	5	167	107	160
	Mean	167	137	201
Vol, cc/24 hr	1	957 (975)	1109 (1046)	1180 (1482)
	2	988	1042	976
	3	1673	1548	2042
	4	827	984	1730
	5	2148	2230	2454
	Mean	1319	1383	1676

* This subject was also observed after cortisone acetate orally, the results of which are tabulated in parentheses for comparison purposes only. They are not included in the means.
Values are means of pooled data for each individual by period.

B. *Urine volume and electrolyte studies.* (Table II) (Values are means of pooled data for each individual by period.) Sodium excretion was reduced during cortisone acetate administration in 4 or 5 individuals, and was increased in the post-treatment period in all subjects. Chloride excretion was uniformly reduced during treatment and increased after drug administration. Potassium excretion increased in 2 of the 5 individuals during cortisone acetate administration. Urine volumes were unaffected by cortisone administration,

TABLE III. Evans Blue Space, Thiocyanate Space and Body Weight during Intramuscular Administration of Cortisone Acetate.

	Subject	Pre-treatment* period	Treatment† period	Post-treatment‡ period
Evans blue space, cc	1	2730 (3330)§	3240 (3530)§	3310
	2	2970	3890	3240
	3	2330	3150	
	4	3860	3520	3520
	5	3520	3680	3930
	Mean	3082	3496	3500
Thiocyanate space, cc	1	15530 (16250)	18270 (17430)	18560
	2	17200	18770	17920
	3	14650	18700	
	4	18320	21590	18790
	5	18270	19580	20600
	Mean	16794	19382	18968
Body wt, kg	1	71.6 (70.2)	73.5 (71.6)	73.7
	2	81.6	82.0	81.6
	3	73.7	73.5	
	4	70.5	72.6	69.5
	5	67.5	68.4	69.3
	Mean	73.0	74.0	73.5

* Determined just before cortisone administration begun.
† " " " last dose of cortisone.
‡ " 2-5 days after therapy was terminated.
§ This subject was also observed after cortisone acetate orally, the results of which are tabulated in parentheses for comparison purposes only. They are not included in the means.

although in 3 individuals there was an elevation in the post-treatment period. Since water was ingested *ad libitum* the meaning of the urine volume changes is uncertain.

C. *Evans blue space, thiocyanate space, and body weight.* (Table III). (Individual values are shown.) Evans blue space increased in 4 of 5 instances during the intramuscular administration of cortisone acetate and in the subject receiving cortisone orally. (Pretreatment values on this same subject performed twice under identical conditions of diet and salt intake, however, differed by 600 cc). Post-treatment figures revealed no consistent pattern. Thiocyanate space increased in every instance during drug administration including the one experiment with oral administration. The post-treatment response was not consistent. Small increases in body weight were noted to drug administration in all experiments except in subject No. 3 whose weight dropped 0.2 kg. The post-treatment response varied.

Discussion and conclusions. The data indicate that normal subjects given cortisone acetate respond in a similar manner to that observed in patients: salt and water retention occurs and serum electrolyte concentration and serum osmolarity are not appreciably altered(12-14). The responses noted here in Evans blue space are similar to the variable results seen in subjects with disease during cortisone administration(15-19). The uniform increase in thiocyanate space is not an unexpected finding since evidence of chloride retention was uniformly noted and since the thiocyanate space empirically parallels changes in chloride space(20-21). The weight gain noted by most of the subjects was considered to represent changes in total body water since the diets, including salt intake, were at the usual daily level for all subjects and the experiments were of brief duration. These data do not make it possible to separate salt and water retention from endogenous shifts of cell water to extracellular space.

---

1. Heller, V. G., and Paul, H., *J. Lab. and Clin. Med.*, 1934, v19, 777.

2. Evelyn Photo-Electric Colorimeter, Bull. No. 460, Rubicon Co., 29 North St., Philadelphia, Pa.

3. Wallace, W. M., Holliday, M., Cushman, M., and Elkinton, J. R., *J. Lab. and Clin. Med.*, 1951, v37, 621.

4. Hawk, P. B., Oser, B. L., and Summerson,

W. H., *Practical Physiological Chemistry*, The Blakiston Co., Philadelphia, 1951, pp 575-577.

5. Kowalski, H. J., and Rutstein, D. D., unpublished observations, 1949-1951.

6. Crawford, J. D., and Nicosia, A. P., *J. Lab. and Clin. Med.*, 1952, v40, 907.

7. Rutstein, D. D., Thomson, K. J., Tolmach, D. M., Walker, W. H., and Floody, R. J., *J. Clin. Invest.*, 1945, v24, 11.

8. Gibson, J. G., II, and Evelyn, K. A., *J. Clin. Invest.*, 1939, v125, 142.

9. Crandall, L. A., Jr., and Anderson, M. X., *Am. J. Digest. Dis. and Nutrition*, 1934, v1, 126.

10. Scheinberg, J. H., and Kowalski, H. J., *J. Clin. Invest.*, 1950, v29, 475.

11. Eder, H. A., *Determination of Thiocyanate Space: Methods in Medical Research*, Chicago, Yearbook Publishers Inc., 1951, v4, 48.

12. Thorn, G. W., Forsham, P. H., Bennett, L. L., Roche, M., Reiss, R. S., Slessor, A., Flink, E. B., and Somerville, W., *Tr. A. Am. Physicians*, 1949, v62, 233.

13. Perera, G. A., Pines, K. J., Hamilton, H. B., and Vislocky, K., *Am. J. Med.*, 1949, v7, 56.

14. Sprague, R. G., Power, M. H., Mason, H. L., Albert, A., Mathieson, D. R., Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F., *Arch. Int. Med.*, 1950, v85, 199.

15. Finch, S. C., Crockett, C. L., Jr., Ross, J. F., and Bayles, T. B., Proc. 2nd Clin. ACTH Conf., vII, *Therapeutics*, 589-598, The Blakiston Co., 1951.

16. Jager, B. V., Brown, H., and Nickerson, M., *J. Lab. and Clin. Med.*, 1951, v37, 431.

17. Levitt, M. F., and Bader, M. E., *Am. J. Med.*, 1951, v11, 715.

18. Copelman, W. S. C., Savage, O., Bishop, P. M. F., Dodds, E. C., Kellie, A. E., Steward, J. W., Glyn, J. H. H., Henly, A. A., and Tweed, J. M., *Brit. M. J.*, 1952, v1, 397.

19. Perera, G. A., Fleming, T. C., Pines, K. L., and Crymble, M., *J. Clin. Invest.*, 1950, v29, 739.

20. Winkler, A. W., Elkington, J. R., and Eisenman, A. J., *Am. J. Physiol.*, 1943, v139, 239.

21. Kaltreider, N. L., Meneely, G. R., Allen, J. R., and Bale, W. F., *J. Exp. Med.*, 1941, v74, 569.

Received July 9, 1953. P.S.E.B.M., 1953, v83.

Effect of Stress upon the Healing of Wounds in Rats.* (20495)

JAMESON L. CHASSIN, HECTOR A. MCDOUGALL, MALCOLM MACKAY, AND S. ARTHUR LOCALIO. (Introduced by John H. Mulholland.)

From the Department of Surgery, New York University Post-Graduate Medical School.

Although the phenomena related to stress have been intensively studied in the past few years, the effect of stress upon the healing process has never been clearly determined. Stress results in increased secretion of adrenal cortical hormones(1). The administration of ACTH or cortisone, in sufficient dosage, inhibits the rate of healing(2,3). Whether or not the increased endogenous secretory activity of the pituitary-adrenal axis in response to stress can depress the rate of healing of an experimental wound, is not established.

Studies concerning the effect of stress upon healing have led to contradictory conclusions. It has been reported(4,5) that if 2 wounds are produced in an animal at different times, the second wound heals more rapidly than the first. On the other hand, it has also been reported(6) that a skin defect, made one or 2 days following a procedure such as gonadectomy, healed more slowly than similar wounds made some time after the operative intervention. Intercurrent infections, located some distance from healing wounds, were observed to slow the rate of healing in a few animals (7). Intramuscular injections of an irritant, sodium ricinoleate, reportedly reduced the rate of healing in dogs on the 7th and 10th postoperative days(8).

In an attempt to clarify the relationship between pituitary-adrenal function and the healing process, a series of experiments was begun in this laboratory in 1951 utilizing the bursting pressure of standard laparotomy

---

* Supported by a grant under contract with the Medical Research and Development Board, Department of the Army. (Contract No. DA-49-007-MD-162).

wounds in rats as an index of the rate of healing.

Method. The technic employed here is a modification of our previously reported method(9) which is objective and withstands statistical analysis. Young Wistar rats, weighing 180-200 g, were submitted to laparotomy with aseptic technic under ether anesthesia. A 3 cm right rectus incision was made and repaired with 6 interrupted 00000 silk sutures through the entire thickness of the abdominal wall. The skin was then sutured with continuous No. 38 stainless steel wire.

On the fifth postoperative day, the animals were again anesthetized with ether. The skin was carefully dissected away from the wound, and the silk sutures removed. Then, through a small stab incision in the left lower quadrant a rubber balloon was inserted into the peritoneal cavity. The balloon was then slowly inflated with air. The pressure of air necessary to burst the abdominal incision was recorded. The end-point has generally been quite sharp and simple to determine. At the conclusion of the wound test, the animals were autopsied. The adrenal glands were carefully dissected out and weighed promptly on a Roller-Smith torsion balance.

Eleven series of observations were made. (Table I) I: Skin incision 6 cm in length over the back, sutured with continuous fine wire 4 days prior to laparotomy. II: Excision of a 6 by 3 cm patch of skin from the back, done 4 days prior to laparotomy. III: Fractures of the femur, knee joint, tibia and fibula bilaterally, produced 4 days pre-laparotomy. IV: Turpentine injection, 1.0 ml, subcutaneously, given in one dose 4 days prior to laparotomy. V: Turpentine, 0.5 ml injections daily beginning 9 days pre-laparotomy and continuing until day of sacrifice. VI: Burn, second degree, 25% of body surface (back), at 73°C for 30 seconds, 4 days pre-laparotomy. VII: Refrigeration at 4-6°C for 4 days prior to laparotomy and continuing until day of sacrifice. The animals were removed from refrigeration only for the duration of the laparotomy procedure. VIII: Epinephrine injections, 0.2 mg daily beginning the day of laparotomy and continuing until day of sacrifice. IX: dl-Thyroxine injections, 0.5 mg daily, beginning 6 days pre-laparotomy. X: Cortisone acetate (Cortone, Merck) injections, 1.0 mg twice daily, beginning 4 days pre-laparotomy. XI: ACTH (Acthar Gel, Armour) injections, 4 units twice daily, beginning 4 days pre-laparotomy. The skin incisions, excisions, burns and fractures were all produced under ether anesthesia.

All animals were maintained in individual cages on an *ad libitum* intake of Purina Laboratory Chow and water. Despite the various injuries, the average body weight of the various groups of rats either increased or remained unchanged during the 10-day experimental period.

Results. The results are summarized in Table I. With the exception of the skin-incision and the single-dose-turpentine stresses, in every case there is a statistically significant depression of the bursting pressure of the fifth day healing wounds. There is a concomitant increase of the adrenal weight in each type of stress in which this factor was studied, as compared with the weight of the adrenal glands in non-stressed laparotomized control rats.

In an attempt to determine for what period of time after initiation of stress this inhibition of healing is manifest, several types of stress were produced at variable intervals of time prior to laparotomy. These results are summarized in Table II. It is apparent that skin incision did not significantly depress the bursting pressure of 5-day wounds at any time interval studied. On the other hand, skin-excision-stress, initiated the day of laparotomy or 4 or 9 days prior to laparotomy, did result in impaired healing. Inhibition of healing did not occur when this type of stress was initiated 15 to 30 days prior to laparotomy. The skin excision wounds were usually healed by the 30th day. When multiple fractures were employed as the stress, depression of the fifth day bursting pressure persisted even 45 days after the fractures were produced.

Discussion. Our results are not in agreement with those of Sandblom(4) and of Young, Fisher, and Young(5). These authors used skin wounds in their experiments. With the use of skin wounds, it is well known that numerous local factors may affect the result,

TABLE I. Magnitude of Stress-Induced Inhibition of Healing as Determined in 5-Day Laparotomy Wounds Following Different Types of Stress.

Nature of stress	No. of rats	Adrenal wt, mg/100 g B.W.	Mean bursting pressure ± S.E.	Statistical analysis*		
				n	t	P
Non-stressed control	27	8.6 ± .2	75.4 ± 2.2	—	—	—
Skin incision, 4 d. pre-lap.	10	9.4 ± .3	66.2 ± 3.7	35	2.08	N.S.
Skin excision, 4 d. pre-lap.	34	10.0 ± .3	60.0 ± 1.5	59	5.25	<.01
Fractures, 4 d. pre-lap.	11	—	60.7 ± 3.4	36	3.53	<.01
Turpentine, 1 cc, 4 d. pre-lap.	10	—	79.0 ± 3.1	35	—	N.S.
Turpentine, .5 cc o.d., 9 d. pre-lap.	11	—	60.9 ± 2.8	36	3.67	<.01
Burn, 4 d. pre-lap.	11	10.0 ± .3	61.3 ± 2.3	36	3.70	<.01
Refrigeration, 4 d. pre-lap.	11	12.5 ± .5	55.3 ± 4.1	36	4.70	<.01
Epinephrine .2 mg, o.d. post-lap.	12	10.8 ± .4	61.0 ± 3.2	37	4.21	<.01
Thyroxine, .5 mg, 6 d. pre-lap.	9	11.6 ± .6	60.7 ± 2.9	34	3.47	<.01
Cortisone, 1.0 mg b.i.d., 4 d. pre-lap.	10	5.8 ± .3	60.0 ± 2.6	35	5.26	<.01
ACTH, 4 u b.i.d., 4 d. pre-lap.	6	15.3 ± .4	53.5 ± 4.0	31	4.18	<.01

* Statistical analysis by the "t-test" of Student as described by Fisher(11). Mean bursting pressure considered significantly different from control when $P < .04$.

S.E. = Stand. error of the mean. N.S. = Not significant.

TABLE II. Effect on Healing of Stress Initiated at Varying Intervals of the Time Prior to Laparotomy.

	Mean bursting pressure	Diff. from control	Statistical analysis		
			n	t	P
Control	75.4 ± 2.2	0	—	—	—
Skin incision					
At lap.	72.7 ± 5.3	2.7	34	—	N.S.
4 d. pre-lap.	66.2 ± 3.7	9.2	35	2.08	.05
9 d. " "	70.9 ± 2.9	4.5	38	1.18	N.S.
Skin excision					
At lap.	63.5 ± 3.7	11.9	37	2.84	<.01
4 d. pre-lap.	60.0 ± 1.5	15.4	59	5.25	<.01
9 d. " "	56.9 ± 2.0	18.5	59	5.25	<.01
15 d. " "	70.6 ± 2.5	4.8	43	1.4	N.S.
30 d. " "	68.9 ± 3.5	6.5	33	1.4	N.S.
Fractures					
At lap.	57.6 ± 3.6	17.8	34	4.04	<.01
4 d. pre-lap.	60.7 ± 3.4	14.7	36	3.53	<.01
9 d. " "	60.0 ± 2.6	15.4	40	4.32	<.01
15 d. " "	59.6 ± 3.3	15.8	35	3.76	<.01
30 d. " "	52.0 ± 2.3	23.4	34	5.6	<.01
45 d. " "	65.9 ± 1.8	9.5	39	2.76	<.01

a fact which is emphasized by Sandblom. This may be an explanation for the discrepancy. In no instance in our study did any group of animals demonstrate a mean bursting pressure, significantly greater than that of the normal control rats. This is in agreement with current views regarding the healing process as summarized by Harvey(10).

The duration of the period of stress-induced inhibition of healing appeared to depend upon the nature and the magnitude of the stress. With multiple fractures, healing was depressed 45 days after the fractures were produced. Actually, bony union of the fractured femoral shafts had not occurred in many cases by this time. Skin-excision-stress did not result in depression of the bursting pressure of laparotomy wounds if the skin excisions were made 15 days or more before laparotomy.

Two possibilities suggest themselves to explain the stress-induced inhibition of healing. First, there may be competition between the repair process in the area of trauma (fractures or skin wounds) and the healing laparotomy wound for certain, as yet undetermined, metabolites which are essential to the healing process. Second, the induced stress may result in sufficient stimulation of pituitary-adrenal activity so that the increased endogenous secretion of adrenal cortical hormone results in inhibition of the rate of healing. The presence of hypertrophied adrenals following severe stress would favor the second hypothesis.

Summary. 1. A variety of situations, calculated to provide a chronic "stress" stimulus, have been studied in order to determine the effect of stress upon the bursting pressure of standard laparotomy wounds in rats. 2. With the exception of mild stress (skin incisions, single turpentine injection), in each case a depression of the bursting pressure of fifth day laparotomy wounds was observed. This was accompanied by increases in the weight of the adrenal glands at autopsy. 3. The duration of this period of stress-induced depression of healing appeared to vary with the magnitude and the nature of the stress stimulus.

1. Selye, H., *The Physiology and Pathology of Exposure to Stress*, Montreal, *Acta Endocrinologica*, 1950.

2. Baker, B. L., and Whitaker, W. L., *Endocrinol.*, 1950, v46, 544.

3. Ragan, C., Howes, E. L., Plotz, G. M., Meyers, K., and Blunt, J. W., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 718.

4. Sandblom, P., *Acta Chir. Scandinav.* (Supp.), 1944, v90, 89.

5. Young, J. S., Fisher, J. A., and Young, M., *J. Path. and Bact.*, 1941, v52, 225.

6. Clayton, B. E., and Prunty, T. T. G., *J. Endocrinol.*, 1951, v7, 363.

7. Carrel, A., *Compt. Rend. Soc. Biol.*, 1924, v90, 333.

8. Sanders, G. A., and Garrison, W. S., *Arch. Surg.*, 1943, v46, 40.

9. Localio, S. A, and Chassin, J. L., *Surg.*, 1952, v32, 39.

10. Harvey, S. C., *Surg.*, 1949, v25, 655.

11. Fisher, R. A., *Statistical Methods for Research Workers*, Edinburgh, Oliver and Boyd, 1938.

Received July 9, 1953. P.S.E.B.M., 1953, v83.

Hafnium Complexes for Biological Investigation.* (20496)

ETTA MACDONALD† AND CARL TABB BAHNER.‡ (Introduced by G. A. Andrews.)

From the Medical Division, Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tenn.

A hafnium sodium mandelate complex has been used in a recent study of the distribution and excretion of hafnium in the rat(1). Hf^{181} is one of the relatively small number of readily available isotopes which have a half-life at all suitable for internal irradiation by deposition in the tissues(2). It therefore seemed important to learn whether it is practical to obtain substantially different distribution patterns using other complexing agents. Other workers have shown that the distribution patterns of colloids can be altered by variation in particle size(3,4) and that the excretion pattern of lanthanum can be changed by the use of chelating agents(5). Another reason for interest in other hafnium complexes is the fact that the mandelate preparations are often visibly cloudy at pH 7-8, while the other complexes described subsequently are more

---

* Work done under contract with the United States Atomic Energy Commission.

† University of Texas Medical Branch, Galveston, Texas.

‡ Carson-Newman College, Jefferson City, Tenn.

satisfactory in that they are more consistently clear.

Sodium catechol-3,5-disulfonate is the agent used to prepare the well known antimonial drug, fuadin. This complexing agent has been found to prevent formation of more than a trace of visible precipitate at pH 7-8 when as little as one mole was used for each 2.5 g atoms of hafnium. A hafnium oxychloride solution containing approximately 9 mg Hf/ml was prepared from irradiated hafnium oxide. A 10 ml portion of this solution was mixed with 0.492 g sodium catechol disulfonate, sodium hydroxide was added until the pH was approximately 7, and the clear solution was diluted to 25 ml. Ratio: 3 moles sodium catechol disulfonate to 1 g atom hafnium.

Sodium citrate, used in the ratio of 2 moles per g atom of hafnium, produced a solution which remained clear at pH 7 but began to become cloudy about pH 8. A solution containing Hf^{181} was prepared by adding 10 ml of the hafnium oxychloride solution to a solution of 0.252 g of $Na_3C_6H_5O_7 \cdot 2H_2O$ in 3 ml of water, adding sodium hydroxide to a pH of 7, then diluting to 25 ml. The resulting solution appeared clear. Ratio: 1.7 moles sodium citrate to 1 g atom of hafnium.

Sodium gluconate formed a clear preparation containing as little as 1.63 moles of sodium gluconate per g atom of hafnium, but an 8:1 ratio was chosen for preparing a complex of Hf^{181}. To 2.0 ml of freshly prepared 2 M sodium gluconate solution were added 10 ml of the hafnium oxychloride solution, then sodium hydroxide to pH 7, and water to a final volume of 25 ml.

For comparison a hafnium mandelate complex was prepared from the same batch of hafnium oxychloride solution used for the preceding preparations, by adding 10 ml of the solution to 0.550 g mandelic acid, adding sodium hydroxide and water to pH 7, and diluting to 25 ml.

TABLE I. Distribution of Hf^{181} in Animals Which Received Hafnium Sodium Catechol Disulfonate Complex.

Rat No.	Specimen	Counts/min./g
1	Portion of left lobe of liver	5.3×10^3
1	" " right " " "	4.0×10^3
1	Portion at junction of lobes of liver	13.3×10^3
2	Portion of left lobe of liver	8.3×10^3
2	" " right " " "	4.6×10^3
2	Portion at junction of lobes of liver	11.6×10^3

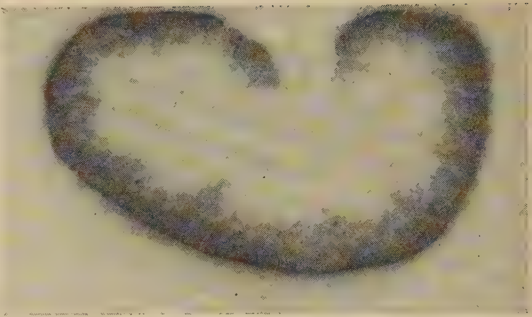


FIG. 1. Autoradiogram of kidney of dog showing uneven distribution of $hafnium^{181}$.

White, male, Wistar-stock rats were used for distribution experiments. A hypodermic needle attached by a short rubber tube to a syringe containing normal saline solution was inserted into the tail vein of each rat under ether anesthesia. Through another needle, 0.5 ml of the radioactive complex was injected into the rubber tube and washed into the blood stream with a small amount of the saline solution. Care was taken that the total volume injected should not exceed 2.5 ml. Since the total amount of radioactive material in each injection was less than 100 μc, it was not believed that radiation affected the distribution pattern significantly. After 96 hours each animal was lightly anesthetized by intraperitoneal injection of nembutal, a blood sample was withdrawn, and the animal was perfused with normal saline solution. The animals were dissected and the weighed organs were dissolved by heating with concentrated nitric acid. The solution was made up to a known volume, was shaken vigorously to disperse the undissolved fatty material, and a 10 ml aliquot portion was placed in a petri dish. The radioactivity was then measured by the use of a thin mica window Geiger-Mueller tube in a lead walled, vertical counting chamber.

In order to minimize the difficulties caused by undissolved fatty material, the entire solution was diluted to only 10 ml and taken for counting whenever possible. The liver was

TABLE II. Hf^{181} Distribution Ratios* 96 Hr after Injection.

Complex	No. of animals	Liver/femur		Kidney/femur		Spleen/femur		Heart/femur	
		Range	Median	Range	Median	Range	Median	Range	Median
Catechol disulfonate	12	.83 - 5.2	1.9	1.3 -13.4	6.2	.78 - 3.7	2.05	.28 -.98	.53
Gluconate	14	.042- .30	.14	.15- 2.3	.79	.077- .70	.18	.018-.11	.066
Citrate	8	.20 - 1.21	.53	.29- 2.1	.52	.016- .40	.14	.023-.10	.051
Mandelate	12	1.6 -18	5.8	.69- 2.1	1.35	2.7 -12.1	5.7	.04 -.9	.32

* Based on counts/g of tissue.

usually minced into very small pieces and a representative sample was taken for digestion and counting. It was not found satisfactory to digest a single strip of the liver as a sample because of the variations in uptake within a single organ as illustrated by the examples shown in Table I. The uneven distribution of Hf^{181} in another organ is illustrated in Fig. 1.

Rather wide variations were observed within the distribution patterns for each of these complexes, but in spite of this fact some striking differences between complexes were apparent. Several of these are illustrated in Table II summarizing distribution ratios at 4 days after injection. The gluconate and citrate complexes behaved very much alike, but the lowest liver/femur ratio in the catechol disulfonate series was nearly 3 times the highest liver/femur ratio in the gluconate series. The liver/femur ratio was even higher in the mandelate series. The kidney/femur ratio was highest in the catechol disulfonate series, while the spleen/femur ratio was highest in the mandelate series.

In all series the concentration of hafnium in the femur was larger than in the heart, but the median heart/femur ratio for the mandelate series was 0.31 while for the catechol disulfonate, citrate, and gluconate series the median figures were 0.53, 0.053, and 0.075. The uptakes in both heart and leg muscle were low, but the uptake in the heart was frequently several times as great as in the leg muscle. The median ratios were: for citrate 2.9, for gluconate 3.0, for mandelate 6.5, for catechol disulfonate 5.9. The median ratios of counts per g of adrenal gland/femur were 2.1 in the mandelate series, and 1.2 in the catechol disulfonate series.

A total of 45 additional rats were used in the study of the distribution patterns for the 3 new complexes at periods from one hour to 16 days after injection. While the data suggest that some gradual change in distribution ratios may occur with time, the evidence is not conclusive. The median ratios shown in Table II would have been little different if the data for the other periods had been included. There was, however, evidence that the concentration of hafnium in the blood decreased rapidly and that excretion of hafnium in the urine occurred much more rapidly during the first few hours than later. A number of the experimental animals were placed in metabolism cages immediately after injection and a conventional apparatus was used to collect urine and feces separately. The activity found in the feces was low. Of 5 rats which received the mandelate complex, none excreted over 8% of the injected dose in the urine within 24 hours, while 9 of 12 rats which received the gluconate and 7 of 8 rats which received the catechol disulfonate excreted half or more of the injected hafnium within 4 hours.

The marked differences in the distribution patterns of hafnium administered with different complexing agents suggest the possibility that the usefulness of radioactive isotopes may be increased by directing the localization in tissues through the use of suitable complexing agents.

Summary. The distribution of Hf^{181} in rats was different when different hafnium complexes were administered. The median liver: femur concentration ratios with various complexing agents were in order mandelate >catechol disulfonate >citrate >gluconate. Differences were observed in other organs also.

---

1. Kittle, C. F., King, E. R., Bahner, C. T., and

Brucer, M., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 278.

2. Brucer, M., *The Merck Report,* 1952, v61, 9.

3. Gofman, J. W., *J. Lab. Clin. Med.,* 1949, v34, 297.

4. Dobson, E. L., Gofman, J. W., Jones, H. B., Kelly, L. S., and Walker, L. A., *Ibid.,* 1949, v34, 305.

5. Hart, H., and Greenberg, J., *Proc. Am. Assn. for Cancer Research,* 1953, v1, 23.

Received July 17, 1953. P.S.E.B.M., 1953, v83.

Effect of Cortisone Upon Formation of Liver Glycogen from Glycine.* (20497)

W. C. HESS AND I. P. SHAFFRAN.

From the Department of Biological Chemistry, Georgetown University School of Medicine, Washington, D. C.

Gluconeogenesis from protein in the fasting animal has been established as being mediated by the adrenal cortex(1-4). Hess and Shaffran(5) have studied the rate of liver glycogen formation from glycine in fasted normal rats. The present paper is concerned with the effect of cortisone upon the rate of glycogen formation from glycine in the fasted normal rat.

Experimental. White rats, weighing 100 to 150 g, fasted for 24 hours were fed, by stomach tube, 450 mg of glycine dissolved in 2.5 ml of water. Cortisone acetate in saline suspension (Cortone Merck) was given intramuscularly at the same time as the glycine at 2 dosage levels, 2.5 and 5.0 mg. Controls receiving only cortisone were also studied. At the end of the experimental periods the animals were sacrificed and liver glycogen determined by the method of Good, Kramer, and Somogyi(6). The animals were kept in metabolism cages during the fasting and test periods and urine was collected for total nitrogen determination by the micro-Kjeldahl method. The results are given in Tables I, II, and III; each value is the average of the results from 4 to 6 animals. Duplicate determinations were made on each sample. For comparison the data for the formation of glycogen from glycine alone(5) are included.

TABLE I. Concentration of Liver Glycogen Following Glycine, 5 mg Cortisone, and Glycine plus Cortisone.

Time, hr	1 Glycine	2 Cortisone	3* Cortisone + glycine	4 Change
	%			
2	.25	.23	.025	—.455 (<.01)†
4	.95	1.80	2.50	—.25 (<.10)
6	1.35	1.60	2.60	—.35 (<.01)
12	2.40	1.80	4.60	+.40 (<.01)
14	2.50	2.35	5.15	+.30
16	2.55	2.40	5.20	+.25
24	1.80	1.90	3.70	.0
31	.40	3.80	4.10	—.10
40	.20	4.00	4.40	+.20
48	.20	3.70	3.90	.0

* Column 3—(Column 1 + column 2).

† P values for the change based upon the calculation for t. From 14 hr to 48 hr the changes are not significant.

Cortisone alone produced the increase in liver glycogen observed by many other investigators. The 5 mg dose induced the maximum deposition at 40 hours, the period from 31 to 48 hours giving a fairly uniform value. A peak was found at 16 hours followed by a slight decline and a subsequent marked increase; a similar effect was noted when cortisone plus glycine were given. The 2.5 mg dose produced a maximum at 24 hours, the amounts found at 31 and 48 hours were slightly less. The maximum amount of glycogen formed following the 5 mg dose of cortisone was almost twice that produced by feeding glycine alone and the maximum formed following the 2.5 mg dose was just slightly less than the peak for glycine alone. However, the maximum glycogen value following glycine was reached earlier.

When 5 mg of cortisone was given at the

---

* This study was aided by a Grant from the Council on Pharmacy and Chemistry of the Am. Med. Assn.

TABLE II. Total Liver Glycogen and Urine Nitrogen, 5 mg Cortisone Dose.

Period and treatment	Liver wt, g	Glycogen, %	Glycogen total, mg	Urine nitrogen, mg
2 hr				
Glycine (7)*	5.3	.25	13.2 (3.7)†	12
Cortisone (4)	5.2	.23	12.0	13
Glycine + cortisone (4)	5.5	.25	1.4	10
6 hr				
Glycine (6)	4.8	1.35	64.8 (17.8)	38
Cortisone (6)	5.2	1.60	83.2	28
Glycine + cortisone (6)	5.1	2.60	132.6 (14.7)	35
24 hr				
Glycine (5)	4.3	1.80	77.4 (21.5)	170 (100)‡
Cortisone (4)	5.0	1.90	95.0	120 (110)
Glycine + cortisone (4)	5.6	3.70	207.2 (28)	200 (110)

* No. of animals used to obtain the avg value given.

† % of glycine converted into glycogen. For the glycine plus cortisone period calculation is based on the assumption that the cortisone would have formed glycogen to the same extent as when administered alone.

‡ Nitrogen excretion for the 24 hr fasting period prior to experimental period.

TABLE III. Concentration of Liver Glycogen Following Glycine, 2.5 mg Cortisone, and Glycine plus Cortisone.

	1	2	3*	4
Time, hr	Glycine	Corti-sone	Cortisone + glycine	Change
		%		
6	1.35	.10	1.20	—.25 (<.01)†
12	2.40	.02	1.90	—.56 (<.01)
18	2.25	.60	2.20	—.65 (<.01)
24	1.80	2.10	3.10	—.80 (<.01)
31	.40	1.70	1.80	—.30 (<.10)
48	.20	1.60	2.40	+.60 (<.10)

* Column 3—(Column 1 + column 2).

† P values for the change based upon the calculation for t.

same time as glycine an increased production of glycogen resulted. In general it appears, from the data in Table I, that the amount of glycogen formed following administration of both glycine and cortisone is equal to the sum of the amounts formed when the two are given separately. This statement may not hold in the first 6 hours during which there appears to be some inhibition of glycogen formation, since the sums of the 2 separate experiments were significantly greater than the amounts formed with the combined administration. Engel, Schiller, and Pentz(7) found that when adrenal cortical extract was administered to rats the amount of glycogen formed during the total experimental period of 6 hours while greater than after the lysine alone was less than after the ACE alone, a result similar to that obtained with glycine and cortisone after 2 hours. For the single period of 12 hours the amount of glycogen formed following both cortisone and glycine was significantly greater than the sum of the 2 separate experiments. For all the other periods there were no significant differences.

Since, during the early hours following cortisone plus glycine, there was inhibition of glycogen formation, based on the per cent formed, the absolute amounts of glycogen were also calculated. These data are given in Table II. The results thus calculated show the same trend; at the end of 2 hours the sum of the values obtained with glycine and with cortisone is 25.2 mg, whereas when the 2 were given together only 1.4 mg of glycogen was formed. At the end of the 24-hour period there was a slight excess of glycogen formed when cortisone and glycine were given together, 207.2 mg compared to 172.4 mg for the sum of the 2 given separately. If the theoretical amount of glycogen that can be formed from glycine is calculated and compared with the total glycogen produced there is a gradual increase in utilization and at the end of 24 hours the liver glycogen accounts for 21.5% of the theoretical amount when glycine is given alone and 28.0% when both glycine and cortisone are given. For the 2- and the 6-hour periods glycine alone gives a higher per cent conversion reflecting the inhibition produced by the hormone at these periods.

Urinary nitrogen data of a number of the animals are also given in Table II. For the 24-hour period there is a comparison between the nitrogen excretion for the 24-hour control and the experimental periods. When glycine alone is given, the increase in nitrogen excretion accounts for 83.5% of the glycine nitrogen, and for both glycine and cortisone, 95.6%. Cortisone alone produced a slight increase, 10 mg, over the control nitrogen excretion in the 24-hour period. The values for the 2- and the 6-hour periods can only be compared within the series and it appears that some inhibition of excretion occurred. In both periods the nitrogen values for cortisone plus glycine are somewhat less than for glycine alone.

With the 2.5 mg dose of cortisone the effects are, qualitatively, similar to those with the 5 mg dose; in fact, the early inhibitory effect is more apparent. Up to 18 hours the values for glycine plus cortisone are less than those for glycine alone. In Table III the hourly values of the liver glycogen are given. The cortisone plus glycine failed to produce as much glycogen as the sum of the amounts formed by the 2 compounds administered separately up to 48 hours. The changes as calculated in column 4 are significant at the 1% level in every case except the last two.

Neither dosage of cortisone increased the amount of glycogen formed from glycine. Perhaps under the fasting conditions employed maximum utilization was occurring and no further increase could result from the action of the cortisone. This result could also be interpreted as supporting the suggestion of Engel, Schiller, and Pents(7) that the action of the adrenal cortex on nitrogen metabolism may be on protein rather than on amino acids.

Summary. 1. Cortisone produced an increase in liver glycogen of fasted normal rats. After a 5 mg dose the peak production period is from 31 to 48 hours, after a 2.5 mg dose the peak is lower and is reached earlier. 2. Glycine given at the same time as the cortisone increased the liver glycogen content. At the 5 mg cortisone dose, after 12 hours, the amount formed was equal to the sum of the amounts formed when each of the substances was given separately. During the earlier periods there was an inhibition of glycogen formation. At the 2.5 mg cortisone level the same results were obtained but to a lesser degree. 3. The excretion of urinary nitrogen reflected the change in liver glycogen in that during the early periods the nitrogen excretion was somewhat less than during the control periods, but within 24 hours the extra urinary nitrogen accounted for 95.6% of the glycine nitrogen.

---

1. Britton, S. W., and Silvette, H., *Am. J. Physiol.,* 1932, v100, 701.

2. Long, C. N. H., Katzin, B., and Fry, *Endocrin.,* 1940, v26, 309.

3. Lewis, R. A., Kuhlman, D., Delbue, C., Koepf, G. F., and Thorn, G. W., *Endocrin.,* 1940, v27, 971.

4. Lockett, M. F., and Evans, M. M., *J. Endocrin.,* 1951, v7, 357.

5. Hess, W. C., and Shaffran, I. P., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 404.

6. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.,* 1933, v100, 485.

7. Engel, F. L., Schiller, S., and Pentz, E. I., *Endocrin.,* 1949, v44, 458.

Received July 17, 1953. P.S.E.B.M., 1953, v83.

Reproducible Oral Infection Rates in Monkeys with the Mahoney Strain (Type 1) of Poliomyelitis Virus.* (20498)

L. P. GEBHARDT AND J. G. BACHTOLD.

From the Department of Bacteriology, College of Medicine, University of Utah, Salt Lake City.

Reproducible and consistent rates of infection of monkeys with the virus of poliomyelitis given orally have not in the past been successful. A variety of vehicles for suspension of the virus have been tried, including water, physiological sodium chloride, milk and butter, milk-soaked bread, bananas, etc. The virus-vehicle mixtures were either fed by means of a syringe or offered to the monkeys for voluntary consumption(1-6). Bodian and Howe(7) using 4 poliomyelitis virus strains (Lansing, Brunhilde, Kotter, and Per) were unable to infect either *M. mulatta* or cynomolgus monkeys by giving virus orally by means of a syringe. Using the Y-SK strain (Type 2) of poliomyelitis virus, Sabin(8) was able to infect 54% of the monkeys when milk was given prior to oral virus feeding. The virus was given twice daily for 3 days. Of 10 controls receiving no milk and one-half as much virus as above, none developed paralytic poliomyelitis. Subsequent studies by Bodian(9) failed, however, to confirm the above data. In these studies, 5 strains of virus were used (Per, Y-SK, Leon, Mahoney, and Wfd) representing all 3 antigenic types. Infection rates were, however, not consistent, varying from 0 to 50%.

With increasing interest in chemoprophylactic and chemotherapeutic studies in experimental poliomyelitis, and considering the fact that the intracerebral and intranasal routes of infection are too severe to screen compounds, a more suitable method for gastrointestinal infection was highly desirable.

Methods. The Mahoney strain (Type 1)[†] of poliomyelitis virus was used. Cynomolgus monkeys from the Philippine Islands were utilized. In one experiment, bread soaked with a milk and virus mixture was given in regular feeding pans. In the remainder, virus was given by stomach tube attached to a syringe, using a Fr. No. 16 urethral catheter, after 48 hours of fasting. Water was offered *ad lib.* during the fasting period

Results. A comparison was first made on the efficacy of voluntary feeding of bread soaked with a mixture of virus and milk, with the same quantity of virus in milk given by stomach tube. The animals were fasted for 24 hours prior to the virus being given. Normal feeding was resumed 24 hours after the virus. The results presented in Table I show that virus given by stomach tube produces a higher infection rate than by simple *ad lib.* virus feeding. Also the problem of "virus splashing" about the cage is eliminated and more accurate dosages can be given by stomach tube.

An attempt was made to titrate the Mahoney virus by the oral route of infection to determine, if possible, a reproducible PD_{50}[‡] dose of virus. In this group of monkeys, virus was given in milk by stomach tube after 48 hours of fasting to eliminate as much as possible residual food in the small intestines. The results presented in Table II show that repeated daily doses of virus are superior to a single dose.

TABLE I. Comparison of Infection Rates of Monkeys Fed Virus in Milk and Virus-Milk Mixture Given by Stomach Tube.

No. monkeys	Virus	Fate	% paralyzed
4	Fed ad lib 5 ml 20% Mahoney virus in 25 ml milk soaked on half slice of bread	2/4*	50
5	5 ml 20% Mahoney virus in 25 ml milk given by stomach tube	4/5	80

* 2/4 = 2 monkeys developed paralysis out of 4 inoculated.

---

* Aided by a grant from the National Foundation for Infantile Paralysis.

[†] A sample of this virus was kindly supplied by Dr. David Bodian, Johns Hopkins University.

[‡] PD_{50} = Dose of virus producing paralysis in 50% of the animals fed with virus.

The next group of animals was tested for the influence of alkaline chemicals and milk fat upon the rate of infection. All animals fasted for 48 hours. Chemicals, milk, cream, and virus were all given by stomach tube. Market milk was used (homogenized and pasteurized) with a fat content of 3.5%; the market cream used was a commercial product (Half and Half) with a fat content of 12%. The results presented in Table III suggest that milk fat rather than alkaline chemicals protects the virus during its sojourn in the stomach.

From the data obtained above (Table III) it was decided to determine whether the amount of milk fat (cream) influenced infection rates, when virus and cream mixtures were given by stomach tube. Thus, Half and Half (12% fat), table cream (18% fat), and whipping cream (33% fat) were used. Virus-cream mixtures were stirred with a glass rod attached to an electric motor, the speed being adjusted so as to prevent frothing. The virus-cream mixture was kept in an ice bath and stirred for 40 minutes. All animals fasted for 48 hours before virus was given.

TABLE II.
Titration of Mahoney Virus by Oral Route.

No. monkeys	Virus	Fate	% paralyzed
5	Single dose. 5 ml 10% virus in 25 ml milk by stomach tube	1/5*	20
4	3 doses same day. 5 ml 5% virus in 25 ml milk by stomach tube	2/4	50
4	3 doses same day. 5 ml 2.5% virus in 25 ml milk by stomach tube	2/4	50

* 1/5 = One monkey developed paralysis out of 5 given virus.

TABLE IV. Effect of Cream on Infection Rate.

No. monkeys	Virus	Fate	% paralyzed
5	5 ml 20% virus in 25 ml half and half cream. Single dose	3/5*	60
5	10 ml half and half cream 1½ hr before virus; then 5 ml 20% virus in 25 ml half and half cream. Single dose	0/5	0
5	5 ml 20% virus in 25 ml table cream. Single dose	2/5	40
5	5 ml 20% virus in 25 ml whipping cream. Single dose	2/5	40

* 3/5 = 3 monkeys developed paralysis out of 5 given virus.

The results presented in Table IV suggest that Half and Half cream (12% fat) and virus mixtures is optimal for reproducible infection rates when virus is given orally in a single dose.

In subsequent chemoprophylactic experiments using the Half and Half cream-virus (Mahoney) mixture (5 ml of 20% virus in 25 ml cream) given by stomach tube, 28 out of 50 control monkeys (representing 5 individual experiments) became paralyzed. This is a consistent and reproducible infection rate of 56%. These results are presented in Table V.

Discussion. From the data presented, cream (12% fat)-virus (Mahoney) mixtures given orally in a single dose, by means of a stomach tube, produce consistent infection rates in cynomolgus monkeys. Multiple doses of virus, as carried out by other workers, have yielded not too constant results. Multiple doses given over periods of 2 or 3 days makes

TABLE III. Effect of Alkaline Chemicals and Cream on Infection Rate.

No. monkeys	Treatment	Virus	Fate	% paralyzed
4	Stomach wash with 2% $NaHCO_3$ 5 min. before virus	5 ml 20% virus in 25 ml milk + 250 mg $Al(OH)_3$. Single dose	2/4*	50
5	250 mg $Al(OH)_3$ in 5 ml H_2O one hr before virus	5 ml 20% virus in 25 ml milk. Single dose	2/5	40
5	———	5 ml 20% virus in 25 ml half and half cream. Single dose	3/5	60

* 2/4 = 2 monkeys developed paralysis out of 4 treated.

TABLE V. Reproducible Infection Rates in Cynomolgus Monkeys by the Oral Route of Virus Inoculation.

No. monkeys	Virus 20% single dose of 5 ml Mahoney virus in 25 ml cream (12%)	Fate	% paralyzed
10	Virus given by stomach tube 48 hr after fasting	5/10*	50
10		6/10	60
10		5/10	50
10		7/10	70
10		5/10	50

* 5/10 = 5 monkeys developed paralytic poliomyelitis out of 10 monkeys inoculated.

it difficult to predict the day of infection. In prophylactic as well as therapeutic studies, the day of infection is of considerable importance. Thus virus given orally in one inoculation which produces a consistent infection rate enables one to more nearly predict the time of infection.

Summary. A method of orally infecting cynomolgus monkeys is presented. Consistent and reproducible infections (56%) have been obtained using the Mahoney strain (Type 1) poliomyelitis virus mixed with cream (12% fat) and introducing the virus-cream mixture into the stomach by means of a Fr. No. 16 urethral catheter in a single dose.

1. Kling, C., Levaditi, C., and Lepine, P., *Bull. Acad. de Med. Paris,* 1929, v102, 158.

2. Schultz, E. W., PROC. SOC. EXP. BIOL. AND MED., 1929, v26, 632.

3. Levaditi, C., Kling, C., and Hornus, G., *Compt. rend. Soc. de biol.,* 1933, v112, 43.

4. Vignec, A. J., Paul, J. R., and Trask, J. D, PROC. SOC. EXP. BIOL. AND MED., 1939, v41, 246.

5. Burnet, F. M., *Med. J. Australia,* 1940, v1, 325.

6. Bodian, D., and Howe, H. A., *J. Exp. Med.,* 1945, v81, 255.

7. ———, *Am. J. Hyg.,* 1948, v48, 99.

8. Sabin, A. B., *Am. J. Pub. Health,* 1951, v41, 1215.

9. Bodian, D., *Am. J. Hyg.,* 1953, v56, 78.

Received July 17, 1953. P.S.E.B.M., 1953, v83.

Selective Inactivation of Esterase by Alcohol. (20499)

GEORGE GOMORI AND HÉLIO B. COUTINHO.*

From the Department of Medicine, University of Chicago, Chicago, Ill.

In the course of studies on the distribution of esterases in the organs of the dog, tissues were fixed in 2 different fixatives: a mixture of equal parts of absolute acetone and 95% alcohol, and neutralized 10% formalin, both chilled. Formalin was washed out thoroughly under the tap, and ice cold acetone was used as the first dehydrating agent. Previous experience has shown that failure to wash out the formalin or the use of alcohol for initial dehydration may result in distinct weakening of enzymatic activity. It was soon found that in formalin-fixed tissues the overall intensity of the alpha naphthol acetate reaction(1) was definitely higher and its distribution more uniform than in the acetone-alcohol-fixed ones. The latter often showed a patchy distribution of activity, and the surface layers of the tissues were more intensely positive than the deeper portions. On closer inspection of the slides it became obvious that the activity of some structures remained practically the same, regardless of fixation, while that of other structures such as the hepatic parenchyma, the renal tubules, and the bronchial mucosa became profoundly depressed. Evidently, the acetone-alcohol mixture had a tendency to destroy the enzyme at certain sites.

To determine the nature of the noxious agent, pieces of tissue were fixed in absolute acetone, 90% acetone, absolute alcohol and 95% alcohol. After fixation in acetone, either absolute or 90%, the pictures obtained were almost as good as after formalin. Absolute

---

* W. K. Kellogg Foundation Fellow from the Department of Histology and Embryology, University of Recife, Brazil.

This work has been aided by grants from the Douglas Smith Foundation for Medical Research, University of Chicago, and from the Pathology Study Section, United States Public Health Service.

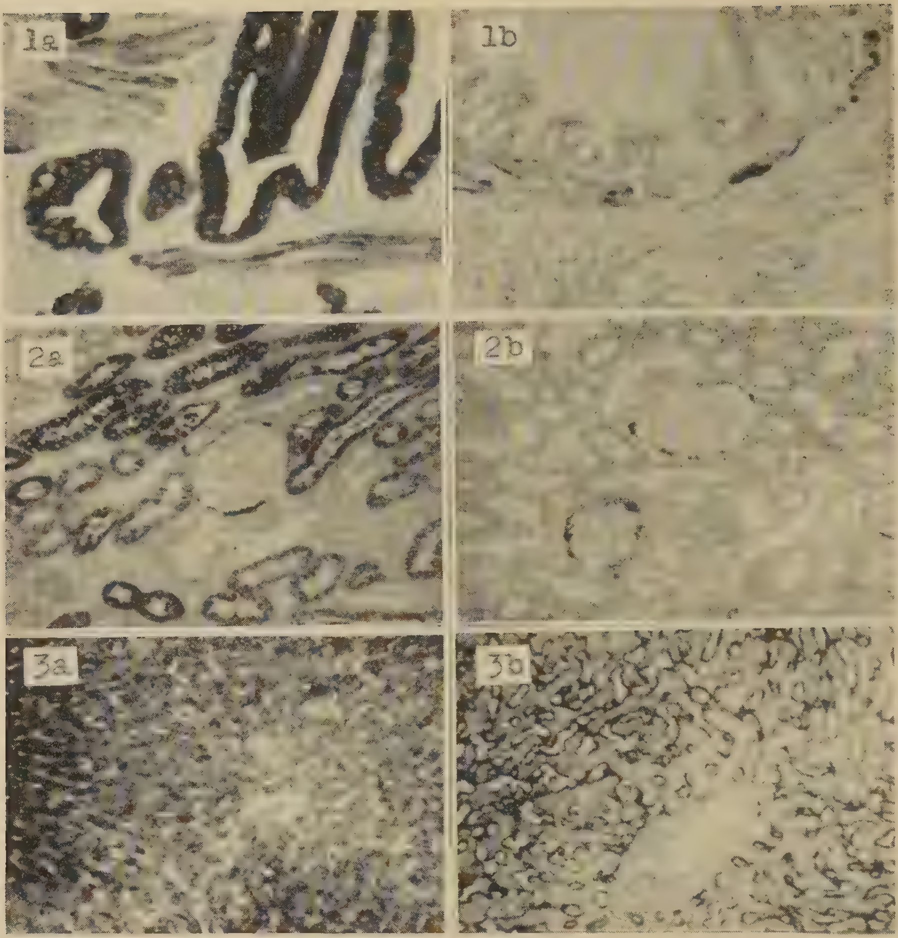


FIG. 1. Lung of the dog. a) Formalin fixation. Bronchial epithelium and smooth muscle stained. b) Alcohol fixation. Epithelium unstained; muscle stained.

FIG. 2. Kidney of the dog. a) Formalin fixation. Renal tubules and Bowman's capsules stained. b) Alcohol fixation. Only Bowman's capsules stained.

FIG. 3. Liver of the dog. a) Formalin fixation. Intense staining of hepatic parenchyma. b) Alcohol fixation. Only the walls of the sinusoids react.

alcohol was distinctly poorer as a fixative and showed some of the effects noted with the acetone-alcohol mixture, while 95% alcohol produced the same effects in an exaggerated fashion. The specific sites mentioned were invariably inactive after fixation in 95% alcohol; on the other hand, other sites such as the walls of the hepatic sinusoids, the mucous neck cells of the stomach, Bowman's capsules in the kidney, etc., lost little if any of their activity (Fig. 1, 2, 3). According to these findings, alcohol, especially in the presence of some water, destroys the enzyme at certain sites.

The question is whether the alcohol-resistant and the alcohol-sensitive localizations of the enzyme should be explained by the presence of different enzymes or by the same enzyme which at certain sites is protected, by an unknown mechanism, against the destructive action of alcohol.

The first possibility was considered the more likely one, especially when it was discovered, by comparing slides stained with the alpha naphthol acetate method with corresponding slides stained with the myristoyl choline technic(2). The alcohol-resistant localizations of the alpha naphthol method coincided remarkably accurately with the structures revealed by myristoyl choline (although the bronchial muscle stained rather faintly with the latter). Following this lead, formalin-fixed sections were incubated in the plain alpha naphthol acetate substrate mixture as before, and other sections in the same mixture with 10^{-5} M eserine added. In the latter group of sections, the activity of the alcohol-resistant sites was completely prevented, and the pictures obtained were the exact reverse of those seen after fixation in 95% alcohol.

The specificity of esterases is a rather complicated problem because of multiple overlapping of substrate preferences(3-5) and of sensitivities to various inhibitors(6). The sensitivity to eserine appears to be the most clearcut differential criterion between aliesterases and cholinesterases(7,8). On this basis, the conclusion that alcohol (95% or weaker) destroys the aliesterase hydrolyzing alpha naphthol acetate in dog organs while it spares a cholinesterase hydrolyzing both alpha naphthol acetate and myristoyl choline, is warranted. In other species this need not be the case since the specificity of esterases varies greatly in different species(6).

Summary. In tissues of the dog, 95% alcohol as a fixative selectively destroys aliesterase while it preserves cholinesterase reasonably well.

1. Gomori, G., *Microscopic Histochemistry*, Chicago, 1952, University of Chicago Press.

2. ———, Proc. Soc. Exp. Biol. and Med., 1948, v68, 354.

3. Mendel, B., and Rudney, H., *Biochem. J.*, 1943, v37, 59.

4. Bovet-Nitti, F., *Experientia*, 1947, v3, 283.

5. Whittaker, V. P., *Biochem. J.*, 1949, v44, XLVI.

6. Gomori, G., and Chessick, R. D., *J. Cell. and Comp. Physiol.*, 1953, v41, 51.

7. Easson, L. H., and Stedman, E., *Biochem. J.*, 1937, v31, 1723.

8. Richter, D., and Croft, P. G., *Biochem. J.*, 1942, v36, 746.

Received July 20, 1953. P.S.E.B.M., 1953, v83.

Immobilization of *Endamoeba histolytica in vitro* by Antiserum Produced in the Rabbit. (20500)

Berwin A. Cole and John F. Kent. (With technical assistance of Victor A. Lopez.)

From the Tropical Research Medical Laboratory, San Juan, P. R., and the Department of Serology, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D.C.

The demonstration that syphilitic infection produces antibody which immobilizes *Treponema pallidum*(1) suggested the possibility that amoeba-immobilizing antibody might appear following infection with *Endamoeba histolytica*. The studies reported herein show that the sera of rabbits inoculated repeatedly with suspensions of cultured *E. histolytica* develop the capacity to immobilize trophozoites of this species *in vitro*.

Materials and methods. All glassware was cleaned with dichromate-sulfuric acid mixture prior to sterilization. A single strain* of *E. histolytica* was employed throughout the studies. The active trophozoites required for immobilization tests were obtained from cultures with a flora of mixed bacteria† on Locke-egg-serum medium(2); a modified(3) Locke's salt solution was used. The best and most consistent yield of motile amoebae was obtained after 20-24 hours incubation at 37°C. A measured volume (0.5 ml) of culture taken from the lowest level of liquid phase was transferred by pipette to a 12 x 75 mm test tube, where it was pooled with aliquots from one or more similar cultures. The tube was then stoppered and replaced in the incubator for an additional 3 hours at 37°C; this served to enhance motility of the trophozoites. During

---

* Strain 200, obtained from the Laboratory of Tropical Diseases, National Microbiological Institute, Bethesda, Md.

† An adventitious combination, the predominating organisms being a Corynebacterium of the xerosis group (sucrose +), an alpha hemolytic streptococcus, and an organism of the achromobacter group.

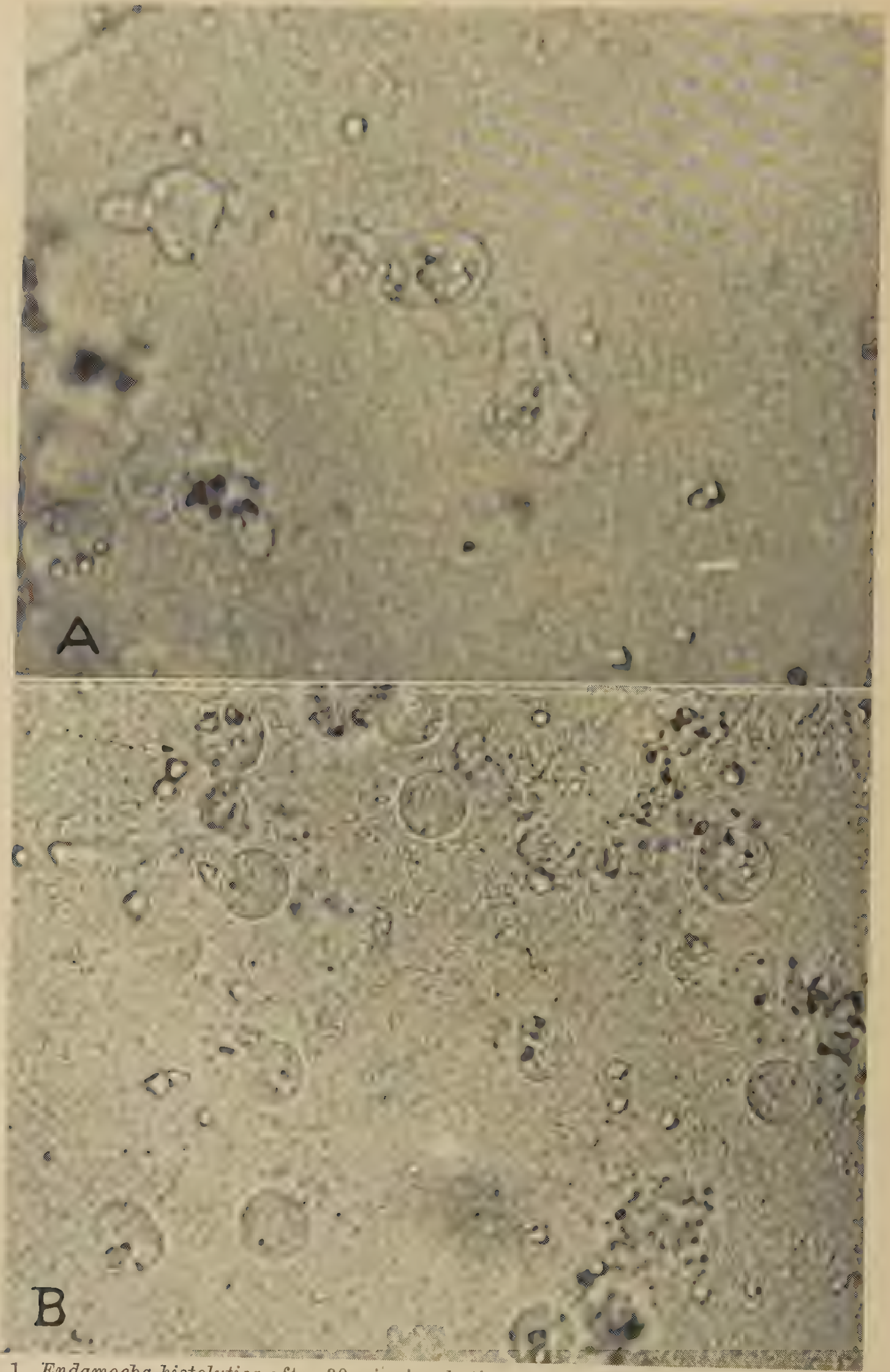


FIG. 1. *Endamoeba histolytica* after 30 min. incubation at 37°C with (A) normal rabbit serum and (B) anti-*E. histolytica* rabbit serum. ×700.

the first half-hour of supplementary incubation the combined cultures were carefully mixed by drawing the material repeatedly into a pipette, and a representative sample was transferred for counting to a Neubauer chamber. Satisfactory culture pools contained at least 2 x 10^5 amoebae per ml; they were standardized at that count by adding a calculated volume of warm (37°C) Locke's-serum-salt solution(2). In the immunization of rabbits it was desired to avoid if possible any stimulation of antibacterial substances, such as might result from use of the amoeba-bacteria culture as inoculum, so advantage was taken of the fact that *E. histolytica* may be cultured also with *Trypanosoma cruzi*(4). The amoeba-trypanosome inocula were prepared from 48-hour cultures showing heavy growth of amoebae. The organisms were washed 3 times with 0.85% NaCl solution and concentrated by centrifugation for 10 minutes at 500 G. They were then suspended in a volume of NaCl solution such that the desired inoculum would be contained in 0.5 ml. Healthy adult rabbits were injected intravenously 3 times weekly for 3 weeks, receiving the organisms from 2 culture tubes at each injection during the first 2 weeks, and those from 4 tubes each time in the third week. The animals were bled by cardiac puncture 7-10 days after the last injection; the sera were separated aseptically and stored at –20°C. Immediately before testing the required volume of serum was heated for 30 minutes in the water-bath at 56°C, after which serial dilutions were made in Locke's salt solution (3). For the immobilization tests, 0.05 ml of the standardized amoeba suspension was mixed on a glass slide with an equal volume of the serum to be tested. The mixture was then covered with a 22 x 22 mm coverslip, which had been rimmed with petrolatum to minimize drying and maintain partial anaerobiasis. The slide was placed finally on an electric warming stage at 37°C, and examined under the low power of the microscope at intervals from 5 minutes to 4 hours. The number of motile trophozoites among the first 25 observed was recorded. In estimating the percentage of amoebae specifically immobilized, the number (A) of motile trophozoites in the test with antiserum was subtracted from the number (B) found motile in the control with normal serum, and the difference multiplied by 100/B.

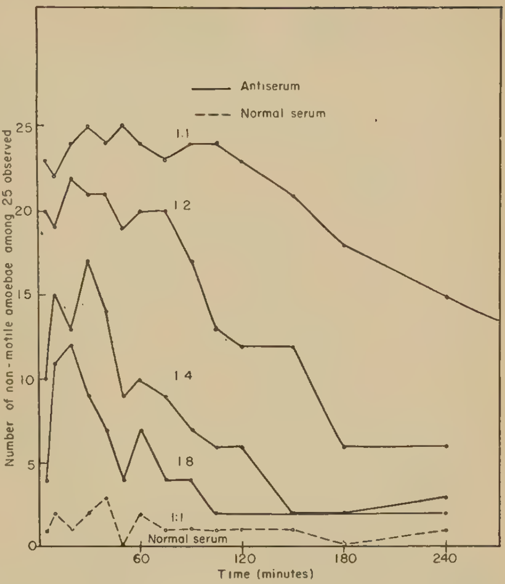


FIG. 2. Immobilization of *E. histolytica* as a function of time and the concentration of rabbit antiserum.

Results. Sera from rabbits inoculated with *E. histolytica* plus *T. cruzi* regularly immobilized amoebae of the same strain cultured with mixed bacteria as commensals. Morphological changes associated with the reaction are illustrated in Fig. 1; when mixed with antiserum, motile trophozoites ceased formation of pseudopodia and assumed a rounded, somewhat contracted form. The results of a representative experiment with serial dilutions of antiserum are given in Fig. 2. The numbers of non-motile amoebae are plotted against the time of incubation in minutes. Included for reference are the numbers of non-motile organisms found concurrently in controls with normal rabbit serum. The number of immobilized trophozoites increased directly with the concentration of antiserum, but varied considerably with the time. Maximal immobilizing effect was observed after 20-30 minutes, the amoebae gradually regaining their activity thereafter. Trophozoites in the controls with normal serum remained actively motile throughout the period of observation. Similar results were obtained with 4 other antisera. The capacity to immobilize *E. histolytica* was not demonstrable in serum from 2 rabbits immunized against *T. cruzi* alone. Evidence of the reproducibility of the immobilization reaction was obtained in repeated titrations of another antiserum; the results appear in Table I. All 7 titrations were per-

TABLE I. Reproducibility of *Endamoeba histolytica* Immobilization Reaction.

Titration No.*	Amoebae immobilized (%) in tests with antiserum (62851-1) diluted in salt sol.				
	1:1	1:2	1:4	1:8	1:16
1	89	71	55	21	3
2	83	74	55	31	17
3	81	67	24	33	10
4	87	63	42	18	8
5	89	64	36	32	16
6	80	69	41	15	13
7	80	65	53	25	15

* Replicate titrations performed during a single day with separate sets of serum dilutions and amoebae from separate cultures.

formed during a single day using separately-prepared sets of serum dilutions and amoebae from separate cultures. The motile amoebae in tests and controls were counted after 30 minutes incubation at 37°C; percentage of specific immobilization was estimated by a method already given herein. Patent discrepancies occurred in individual test mixtures, as in titrations 3 and 5, but the findings otherwise were in good agreement. Two subsequent observations were significant in that they indicated the direction for further standardization, and suggested a practical application of the immobilization reaction. Thus *E. histolytica* cultured with a single bacterial species(5) as commensal could be substituted successfully for the amoebae grown with mixed bacteria. Preliminary tests showed also that the capacity to immobilize amoebae occurs in serum from patients with amoebiasis. In a group of 13 individuals shown to be infected with *E. histolytica* 5 reacted in the immobilization test. The reactive capacity of their sera was relatively low, however, as was that of the rabbit antisera (Fig. 2 and Table I). Serums from a group of 48 normal individuals were without effect upon the amoebae.

Discussion. The immobilization of *E. histolytica* by rabbit antiserum differs in certain essential characteristics from the corresponding reaction between *T. pallidum* and its antibody. In the case of the treponeme the loss of motility appears to be an irreversible one (1). A marked difference in the studies with amoebae was the early recovery of immobilized trophozoites. It appears also that complement (C′), which plays an important role in the reaction with treponemes(1), may not be essential in the immobilization of amoebae. In the present experiments each of the sera had been heated under conditions ordinarily employed for inactivation of labile C′ components, so it could be assumed that the latter at least did not participate in the reaction. Further studies of the nature and kinetics of the amoeba-immobilization phenomenon are clearly indicated. At the same time serious consideration should be given to practical applications of the reaction as a diagnostic aid. Any method which can demonstrate a serologic response to *E. histolytica* may assist in establishing the etiological agent in obscure clinical illnesses. The simplicity and reproducibility of the present technic suggest its early evaluation. Any further studies, moreover, should consider the relationship between complement-fixing antibody in amoebiasis and the capacity to immobilize trophozoites. The demonstration of a distinct immobilizing function, as in the case of the treponematoses, would be especially significant in view of the limited diagnostic usefulness(6) of the complement-fixation test.

Summary. The sera of rabbits inoculated with cultures of *E. histolytica* immobilize the trophozoites of this species *in vitro*. The capacity to immobilize amoebae occurs also in the serum of individuals infected with *E. histolytica*. A technic for demonstrating the immobilization reaction is described, and evidence of its reproducibility is presented.

---

1. Nelson, R. A., and Mayer, M. M., *J. Exp. Med*, 1949, v89, 369.
2. Boeck, W. C., and Drbohlav, J., *Am. J. Hyg.*, 1925, v5, 371.
3. Stone, W. S., *Am. J. Trop. Med.*, 1935, v15, 681.
4. Phillips, B. P., *Science*, 1950, v111, 8.
5. Rees, C. W., Bozicevich, J., Reardon, L. V., and Jones, F., *Am. J. Trop. Med.*, 1942, v22, 581.
6. Hussey, K. L., and Brown, H. W., *Am. J. Trop. Med.*, 1950, v30, 147.

Received July 20, 1953. P.S.E.B.M., 1953, v83.

Effect of Erythromycin, Thiocymetin, and Three Other Antibiotics on *Leptospira icterohemorrhagiae* in the Chick Embryo. (20501)

RICHARD A. ORMSBEE. (Introduced by C. L. Larson.)

From the Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Microbiological Institute, Rocky Mountain Laboratory, Hamilton, Mont.

The leptospiroses form a clinical complex which is world-wide in distribution and has been found in every geographical region of the United States. Only recently has it been realized that the disease is not only widespread in the continent but that the incidence is considerably higher than had been generally known both in animal and man(1).

Penicillin and streptomycin have heretofore been considered to be among the more effective drugs in the treatment of the disease(2,3). Aureomycin has been shown to be effective in terminating the carrier condition in experimentally induced *L. canicola* infections in hamsters and dogs(4). Chloramphenicol has been reported to be ineffective in experimental infections with *L. icterohemorrhagiae* in hamsters and guinea pigs and in eradicating the carrier state in dogs(5). With respect to human leptospirosis, the statement has been made that none of these antibiotics has been shown to be particularly promising as an effective drug for the treatment of clinical cases(5).

It therefore became of interest to examine the effect of two new compounds, erythromycin and thiocymetin, on *Leptospira icterohemorrhagiae,* the causative agent of Weil's disease in man, which along with *L. canicola* and *L. pomona* and other species, causes leptospirosis in both animals and man. This communication reports the relative potencies of erythromycin(6,7), thiocymetin(8,9), chloramphenicol, aureomycin, streptomycin, and penicillin G in terms of their ability to delay the death of chick embryos infected with *L. icterohemorrhagiae.*

Materials and methods. The compounds tested were erythromycin, Dd-threo-2-dichloroacetamido-1-(4-methylsulfonylphenyl)-1, 3-propanediol or thiocymetin, chloramphenicol, aureomycin HCl, the potassium salt of penicillin G, and streptomycin trihydrosulfate.

A serologically typical strain (L204) of *L. icterohemorrhagiae* which was originally isolated from a rat and which is virulent in guinea pigs was employed in all tests.

The tests were carried out by injecting groups of 25 to 30 6-day-old chick embryos via the yolk sac with 0.2 ml aliquots of a suspension of *L. icterohemorrhagiae* of such a concentration that the mean death time in control groups averaged 4.7 to 4.8 days. Twenty-four hours after injection of the organisms, the embryos were similarly injected with aqueous solutions of drug given in 0.5 ml aliquots and in amounts not exceeding the previously determined maximum tolerated dose. All embryos dying within 72 hours after injection of the organisms were discarded and not counted in the test results on the basis that such deaths (which averaged 15%) were due to trauma of injection. The embryos were then candled daily for 13 days, at which time tests were terminated. Embryos still alive on the 13th day were considered dead on the 14th day for purposes of calculation. The difference between the mean death time of the control groups and the treated groups was taken as a measure of the therapeutic effectiveness of the drug.

Experimental results. A summary of the test results is presented in Table I. The standard error of the mean (σ_m) in the control groups ranged from $\pm$ 0.05 to $\pm$ 0.09 days. In the experimental groups σ_m was somewhat larger due to increased scatter of the data. In the tests with all compounds except erythromycin, σ_m was $\lesssim \pm 0.4$ days. In the erythromycin tests, σ_m was $\lesssim \pm 0.6$ days.

As can be seen in Table I, the compounds may be ranked in the following order of effectiveness on the basis of equi-molar dosages: erythromycin >penicillin G >streptomycin >aureomycin >thiocymetin >chloramphenicol.

The data in Table I indicate that erythromycin in the chick embryo was of the order of 8 to 40 times more effective on an equi-molar

TABLE I. Effect of Erythromycin, Penicillin G, Streptomycin, Aureomycin, Chloramphenicol, and Thiocymetin on Mean Death Time of Chick Embryos Infected with *L. icterohemorrhagiae*. MD = mean death time in days.

Compound tested	μg/egg	μM/egg	No. of tests	MD, exp.*	MD, control (σ_m ≦ ± .09)	Delay in MD
Erythromycin	11	.015	1	7.7 (0/23)	4.7	3.0
	22	.03	1	9.9 (0/22)	4.7	5.2
	40	.06	1	≧ 12.3 (5/24)	4.8	≧ 7.5
	80	.11	1	≧ 13.1 (25/31)	4.8	≧ 8.3
Penicillin G	90	.24	1	≧ 9.5 (2/24)	4.7	≧ 4.8
	900	2.40	1	≧ 12.6 (18/24)	4.7	≧ 7.9
Streptomycin	220	.25	1	≧ 7.5 (1/25)	4.7	≧ 2.8
	2200	2.50	1	≧ 11.0 (7/25)	4.7	≧ 6.3
Aureomycin	129	.25	1	6.9 (0/23)	4.7	2.2
	250	.49	4	8.9 (0/140)	4.8	4.1
	515	1.00	1	10.7 (0/21)	4.7	6.0
Chloramphenicol	81	.25	1	5.2 (0/24)	4.7	.5
	808	2.50	1	9.2 (0/22)	4.7	4.5
Thiocymetin	100	.28	2	5.7 (0/53)	4.8	.9

* Figures in parentheses refer to number of embryos alive on 13th day post-inoculation over total number of embryos used in test.

basis than penicillin G, and 17 to 40 times more effective than streptomycin or aureomycin.

Streptomycin and aureomycin in these tests were not significantly different in their effect and both were definitely less effective than erythromycin or penicillin G.

Chloramphenicol and thiocymetin were not significantly different from each other in their effect at equi-molar concentrations. The delay in death afforded by these compounds at doses of 0.25-0.28 μM was probably not significant. Chloramphenicol at a dose of 2.5 μM however, gave a significant delay in mean death of 4.5 days. Thiocymetin could not be tested at a dose higher than 0.28 μM since this is the maximum tolerated dose for chick embryos.

Summary and discussion. Five broad spectrum antibiotics and chemotherapeutic agents have been tested for their relative effectiveness in suppressing *L. icterohemorrhagiae* infections in chick embryos. The drugs may be ranked in the following order of potency: erythromycin >penicillin G >streptomycin >aureomycin >thiocymetin >chloramphenicol. These results indicate that erythromycin is potentially a valuable aid to leptospirosis therapy. This must be tested in experimental animals in comparison with penicillin and streptomycin before any more definite statement can be made.

It is a pleasure to acknowledge the technical assistance of Hazel Parker, Edgar Pickens, and Mary Wicht.

---

1. Reinhard, K. R., *Exp. Parasitol.*, 1953, v2, 87.
2. Brunner, K. T., and Meyer, K. F., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 450.
3. Larson, C. L., and Griffitts, J. J., *Pub. Health Rep.*, 1945, v60, 317.
4. Brunner, K. T., and Meyer, K. F., *Am. J. Vet. Res.*, 1950, v11, 89.
5. Hall, H. E., Hightower, J. A., Rivera, R. D., Byrne, R. J., Smadel, J. E., and Woodward, T. E., *Ann. Int. Med.*, 1951, v35, 981.
6. McGuire, J. M., Bunch, R. L., Anderson, R. C., Boaz, H. E., Flynn, E. H., Powell, H. M., and Smith, J. W., *Antibiotics and Chemotherapy*, 1952, v2, 281.
7. Heilman, F. R., Herrell, W. E., Wellman, W. E., and Geraci, J. E., *Staff Meet. Mayo Clinic*, 1952, v27, 285.
8. Cutler, R. A., Stenger, R. J., and Suter, C. M., *J. Am. Chem. Soc.*, 1952, v74, 5475.
9. Nachod, F. C., and Cutler, R. A., *J. Am. Chem. Soc.*, 1952, v74, 6291.

Received July 20, 1953. P.S.E.B.M., 1953, v83.

Pepsin Secretion and Synthesis *in vitro*.* (20502)

Ramon Villarreal.† (Introduced by Horace W. Davenport.)

From the Department of Physiology, University of Utah College of Medicine, Salt Lake City, Utah.

In vitro preparations of organs can be used to study secretion and synthesis of enzymes, and the purpose of the work reported here was to determine whether pepsin is synthesized by the isolated stomach. Edwards and Edwards (1) measured the secretion of pepsin by the toad gastric mucosa, but because they did not measure pepsin contained in the mucosa the synthesis of pepsin was not observed. Hokin (2) stated he was unable to obtain pepsin synthesis *in vitro*, but he gave no details.

Methods. Stomachs of mice of the C57 Black strain were used. They were prepared by the methods used for studying acid secretion *in vitro* which have been described in detail(3,4). The stomachs were filled to an internal pressure of 25 or 50 cm H_2O with isotonic, phosphate-buffered salt solution containing 20 mM glucose. They were placed in 4 ml of the same solution and incubated with shaking at 38°C under a pO_2 of 3200 mm Hg. After incubation the fluid within the stomach was removed, and its pH was measured. Acid secreted was estimated by multiplying the volume of the fluid by the number of micromoles of acid required to reduce the pH of a unit volume of the solution from its original to its final pH. The pepsin activity of the solution was measured after adjustment to pH 2 by the hemoglobin method of Anson and Mirsky(5). In the course of analysis all pepsinogen was converted to pepsin. The wet weight of the glandular portion of the stomach with the forestomach and the stumps of the duodenum and esophagus trimmed away was obtained. This tissue which consisted of both mucosa and muscularis was extracted by thorough grinding in 0.1 M acetate buffer at pH 4.5, and pepsin content of the extract was measured after adjustment to pH 2. Stomachs not incubated were also extracted. The analytical method was standardized using 2X recrystallized swine pepsin (Cudahy), and the results were expressed as mg pepsin per g wet weight of tissue.

Results. Stomachs were filled to an internal pressure of 50 cm H_2O and incubated for one hour in salt solutions containing various concentrations of carbaminoylcholine. Their rates of pepsin secretion are shown in Fig. 1. At zero drug concentration there is a basal rate of secretion. This basal rate is stimulated in part by distention of the stomach and in part by unknown factors. The maximal rate of secretion occurs at a drug concentration of 0.1 mg %, and at higher drug concentrations pepsin secretion falls toward the basal rate. The response of pepsin secretion to the parasympathomimetic drug closely parallels the response of acid secretion(3) which has a basal rate at zero drug concentration, a maximal rate

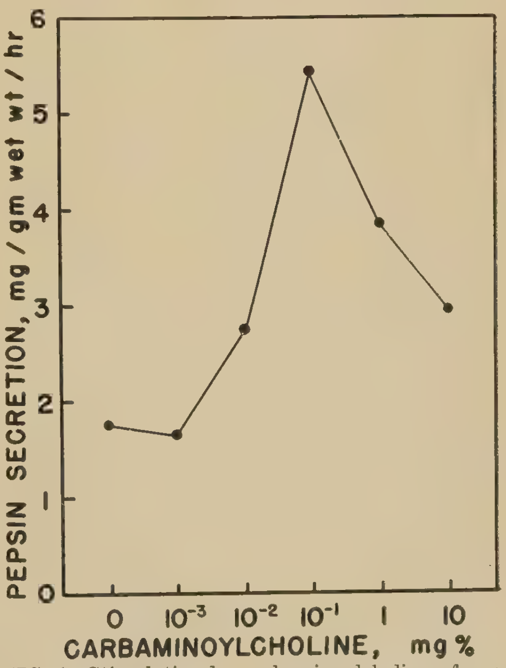


FIG. 1. Stimulation by carbaminoylcholine of pepsin secretion by mouse stomachs *in vitro*. Each point represents the mean of 5 or more observations.

---

* This investigation was supported by a research grant from the Division of Research Grants, National Institutes of Health, Public Health Service.

† Fellow of the Kellogg Foundation.

TABLE I. Pepsin Secreted by or Contained in Mouse Stomachs. (mg pepsin/g wet wt.)

Hr of incubation	Substrates	Total pepsin secreted, mean ± S.E.*	Pepsin in stomach, mean ± S.E.*
0	—	—	12.5 ± .4
1	Glucose	2.9 ± .1	9.7 ± .4
4	"	5.4 ± .3	5.4 ± .3
4	" + casein hydrolysate	—	5.6 ± .3
4	" + amino acid mixture	—	4.7 ± .2

* Stand. error of mean: $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

at 0.1 mg % and which falls off at higher concentrations. In 30 instances in which rates of both acid and pepsin secretion were measured on the same preparation the correlation coefficient between the variables was +0.84.

Atropine sulfate in concentrations of 0.01, 0.1 or 1 mg % has no effect upon the basal rate of pepsin secretion, but when carbaminoylcholine is also present these concentrations of atropine reduce the rate of pepsin secretion to the basal rate but not below it. Histamine in a concentration of 1 mg % increases the rate of pepsin secretion to about 50% above the basal rate.

In order to determine whether pepsin is synthesized *in vitro* the sum of the amount of pepsin secreted and contained in the stomach after incubation was compared with the amount present in unincubated stomachs. Results of these experiments are given in Table I. The pepsin content of stomachs not incubated is given in the first line. Stomachs filled to 25 cm H_2O internal pressure and incubated one hour in the presence of 0.1 mg % carbaminoylcholine and 20 mM glucose contained less pepsin at the end of incubation, but the sum of this pepsin plus that secreted equalled that in the control stomachs. To see whether the total pepsin would increase during a longer period of incubation stomachs were incubated under the same conditions for one hour, emptied, refilled and again incubated for an hour. The process of emptying and refilling was repeated for a total of 4 hours, and the total pepsin secreted and contained in the stomach was measured. The amount secreted in the 4 successive hours was 2.9, 1.2, 0.7 and 0.6 mg per g wet weight. The sum of that secreted and contained in the stomach was less than that present in the control stomachs. The differences can be accounted for by the losses occurring during the process of emptying and refilling. It is clear that no synthesis of pepsin from endogenous precursors occurred. The experiments were repeated with two different amino acid mixtures added to the incubation fluid. These were 0.2% of a tryptic digest of casein and a 0.15% mixture of the 19 amino acids occurring in pepsin(6) made up in the proportion in which they exist in the enzyme. Because it is not possible to use the Anson and Mirsky pepsin method on solutions containing tyrosine and because other pepsin methods are not sufficiently sensitive or accurate, the pepsin secreted in the presence of the amino acid mixtures could not be measured. In neither instance was the amount of pepsin found in the stomach at the end of 4 hours incubation greater than that found when only glucose was the substrate.

Discussion. Mouse stomachs contain an average of 12.5 mg pepsin per g wet weight. The tissue on which the weight was determined is approximately 56% mucosa and 44% muscularis(7). Therefore the pepsin content of the mucosa is about 23 mg per g wet weight. This is much higher than the figure of 6.4 mg per g wet weight for swine fundic mucosa derivable from the figures given by Herriott (8). The dry weight to wet weight ratio of the mouse stomach is 0.25, so that about 9% of the solids of the mucosa is pepsin or pepsinogen.

The conditions under which the stomachs were incubated are optimal for acid secretion (3), but they are not adequate for pepsin synthesis. Pepsin may not be synthesized because the chief cells are damaged by the *in* *vitro* conditions or because the correct precursors were not supplied. The facts that the parietal cells function under these conditions and that the histological appearance of the stomach is good after incubation(9) make it doubtful that the chief cells are seriously injured. Hokin(2) in the same paper in which he notes he was unable to obtain pepsin synthesis shows that a mixture of amino acids or 0.2% casein hydrolysate supports enzyme synthesis by pancreatic slices *in vitro*. The stomach may require more complex substances than amino acids for pepsin synthesis, but there is no evidence indicating the nature of these requirements.

The fact that pepsin synthesis does not occur under the conditions described has an important bearing on the calculation of the efficiency of gastric acid secretion. The oxygen consumption and acid secretion by mouse stomachs have been measured under nearly the same conditions(10). The efficiency of the acid-secreting process in terms of moles of hydrogen ions secreted per mole of oxygen used for acid secretion has been calculated from the observed increases of both variables upon stimulation by 0.1% carbaminoylcholine. The efficiency has been found to be about 2 moles of acid per mole of oxygen. If some of the additional oxygen used after stimulation of the stomach were used for pepsin synthesis rather than for acid secretion the calculated efficiency would be lower than the true efficiency. Because no pepsin is formed, no correction need be applied for the process. However, pepsin secretion is stimulated by the drug, and if any appreciable amount of oxygen is used for pepsin secretion the calculated cost of acid secretion should be corrected for this.

Summary. Pepsin secretion and synthesis by mouse stomachs was studied *in vitro*. There is a basal rate of pepsin secretion, and carbaminoylcholine stimulates additional secretion. At the optimal drug concentration of 0.1 mg % the rate of secretion is 300% of the basal rate. Atropine inhibits the additional secretion caused by carbaminoylcholine but not the basal secretion. Histamine slightly stimulates pepsin secretion. The stomachs were supplied with amino acid mixtures, but no pepsin synthesis could be found. The implications of failure to find synthesis are discussed.

---

1. Edwards, L. E., and Edwards, C. T., *Fed. Proc.*, 1948, v7, 30.
2. Hokin, L. E., *Biochem. J.*, 1951, v48, 320.
3. Davenport, H. W., and Chavre, V. J., *Gastroenterology*, 1950, v15, 467.
4. Davenport, H. W., *Methods in Medical Research*, 1951, v4, 185, Year Book.
5. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1932-33, v16, 59.
6. Northrop, J. H., Kunitz, M., and Herriott, R. M., *Crystalline Enzymes*, 1948, 2nd Edition, Columbia.
7. Davenport, H. W., *Gastroenterology*, 1947, v9, 293.
8. Herriott, R. M., *J. Gen. Physiol.*, 1938, v21, 501.
9. Davenport, H. W., Schneebeli, G. L., and Chavre, V. J., *Fed. Proc.*, 1950, v9, 28.
10. Davenport, H. W., and Chavre, V. J., *Am. J. Physiol.*, in press.

Received July 21, 1953. P.S.E.B.M., 1953, v83

Priming the Anestrous Cat for Reflex Discharge of Pituitary Ovulating Hormone.* (20503)

CHARLES H. SAWYER AND JOHN W. EVERETT.

From the Departments of Anatomy, University of California at Los Angeles and Duke University Medical Schools, and the Investigative Medicine Service, Veterans Administration Hospital, Long Beach, Calif.,

The female cat, like the rabbit, ferret, mink and several other species, does not ordinarily ovulate spontaneously, but only after mating. Copulation in these forms initiates a reflex discharge of ovulating hormone from the adenohypophysis, which in turn leads to rupture of ovarian follicles. Although the nervous pathways involved in this reflex are incompletely understood, Greulich(1) demonstrated that in estrous cats prodding the uterine cervix with a glass rod was an effective stimulus for inducing ovulation.

In studies of neural control of the hypophysis and ovulation, although the rabbit has a number of advantageous characteristics that have resulted in its extensive use, the cat appears to have certain special advantages of its own as an experimental animal. Stereotaxic coordinates of the cat brain are available (2), the brain withstands lesions very well, and the behavorial afterreaction to vaginal stimulation in the estrous cat leaves no doubt that stimulation of higher nerve centers has been achieved. Unfortunately, however, the cat stays in heat for only a few days at a time, and for a long period from July to January(3), it remains completely anestrous. Earlier workers(4-6) using female sex steroids have induced estrous behavior in the anestrous cat without reference to ovulation. Others (7-11) have induced ovulation directly with the use of pituitary and placental gonadotrophins without involving nervous mechanisms. The present problem has been to prime the animal in such a way that neurogenic stimulation of her own hypophysis is necessary to induce ovulation. Only thus can ovulation be considered an indicator of successful stimulation of the hypophysis.

The results are summarized in Table I. Estrogen refers to estradiol benzoate (0.83 mg/ml in oil)† and PMS to equine gonadotrophin (Anteron).† The vaginal stimulus was produced by several thrusts of a blunt glass rod, 5 mm in diameter, to the uterine cervix. Estrus implies that the cat not only raised her pelvis, deflected her tail and treaded in response to prodding the perineum, but that she responded to vaginal stimulation by a typical afterreaction—violent rolling, licking the vulva and rubbing the head and neck on the side of the cage.

Twelve cats received estrogen alone in divided subcutaneous doses totalling less than 0.3 mg over several days. While this resulted in complete behavioral estrus in 9 cats, laparotomy revealed ovarian stimulation in not one animal. Equine gonadotrophin in subcutaneous doses of 50 I.U. each on 2 consecutive days failed to stimulate either estrous behavior or ovulation in 5 cats. Incidentally, higher dosages of PMS (100 I.U.) on 3 successive days induced ovulation in 2 cats, independently of nervous stimulation, confirming Windle's(11) report. Fifteen cats received a standard regime of 0.04 mg estrogen and 50 I.U. PMS for 2 days and 0.08 mg estrogen alone on the third day without ovulating. They generally appeared to be in heat but were not given vaginal stimulation. Five similarly treated animals were subjected to vaginal stimulation without provoking an afterreaction and none ovulated. However, of 16 anestrous cats treated in this way and responding to cervical stimulation with the typical afterreaction, 9 out of 16 ovulated, presumably as a result of reflexly induced discharge of their own pituitary gonadotrophin.

In searching for the reason why the other 7 females did not ovulate, histological sections

---

* These studies were aided by a contract between the Office of Naval Research, Department of the Navy and the University of California at Los Angeles School of Medicine (NR 113-223)

† Kindly supplied by the Schering Corp., New Brunswick, N. J.

TABLE I. Effects of Estrogen and/or PMS in the Anestrous Cat on the Onset of Heat and Induction of Ovulation by Vaginal Stimulation.

No. of cats	Treatment			No. of cats reaching estrus	Ovulation	
	Estrogen	PMS	Vaginal stimulus		No.	%
			Anestrous			
12	+	—	+	9	0	0
5	—	+	+	0	0	0
15	+	+	—	—	0	0
5	+	+	+	0	0	0
16	+	+	+	16	9	56
			Estrous			
10	—	—	+	10	5	50

were made of several of the anovulatory ovaries. The latter contained unusually large follicles, more than 4 mm in diameter in 5 of the 7 cats and actually cystic in 2. The enlarged follicles revealed a thin granulosa, perhaps beyond the stage of responsiveness to ovulating hormone. In the ovulating animals some of the larger follicles luteinized without rupturing. The conditions recall the situation in which follicles overgrown in response to FSH failed to rupture in response to LH(9).

We have recently had the opportunity of observing cats in naturally occurring estrus and we have had similar results with them (Table I). Five out of 10 failed to ovulate in response to vaginal stimulation, and examination of their ovaries revealed similarly hypertrophied, almost cystic ovaries. Others (12,1) have reported that actually mating the cat does not insure ovulation under laboratory conditions. Perhaps left to her own devices in nature the female cat seeks out the male at the appropriate stage in the growth of her follicles, but under laboratory conditions the stimulus may arrive too late.

Estrogen without PMS is an effective priming agent for neurogenic stimulation of ovulation in the rat(13) and the rabbit(14). In anestrous sheep it has also been reported(15, 16) that injection of estrogen will often invoke ovulation. Possibly in the anestrous cat estrogen likewise facilitates hypothalamo-hypophyseal mechanisms, but the ovary cannot respond until growth of its follicles has been promoted by PMS.

Summary. The results indicate that the nervous-pituitary-ovarian conditions of the estrous cat may be simulated by estrogen-PMS priming, and that cats in such artificial estrus may be employed as favorable material in experimental studies on nervous control of the adenohypohysis.

---

1. Greulich, W. W., *Anat. Rec.*, 1934, v58, 217
2. Jiminez-Castellanos, J., *J. Comp. Neur.*, 1949, v91, 307.
3. Dawson, A. B., in Farris, *The Care and Breeding of Laboratory Animals*, Wiley, New York, 1950, Chap. 8, 202.
4. Bard, P., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1939, v19, 190.
5. Maes, J. P., *Nature*, 1939, v144, 598.
6. Dempsey, E. W., and Rioch, D. M., *J. Neurophysiol.*, 1939, v2, 9.
7. Snyder, F. F., and Wislocki, G. B., *Bull. Johns Hopkins Hosp.*, 1931, v49, 106.
8. Bourg, R., *C. R. Soc. de Biol.*, 1933, v114, 562.
9. Foster, M. A., and Hisaw, F. L., *Anat. Rec.*, 1935, v62, 75.
10. Friedgood, H. B , *Am. J. Physiol.*, 1939, v126, 229.
11. Windle, W. F., *Endocrinology*, 1939, v25, 365.
12. Longley, W. H., *Am. J. Anat.*, 1911, v12, 139.
13. Everett, J. W., in Soskin: *Progress in Clinical Endocrinology*, Grune & Stratton, Chap. 7, 319.
14. Sawyer, C. H., *Anat. Rec.*, 1949, v103, 502.
15. Hammond, J., Jr., Hammond, J., and Parkes, A. S., *J. Agric. Sci.*, 1942, v32, 308.
16. Hammond, J., Jr., *J. Endocrinol.*, 1945, v4, 169.

Received July 22, 1953. P.S.E.B.M., 1953, v83

Propagation of St. Louis Encephalitis Virus in Cells of the Ehrlich Ascitic Tumor of Mice.* (20504)

F. S. Cheever and James Dickos.

From the University of Pittsburgh, Graduate School of Public Health, Pittsburgh, Pa.

Koprowska and Koprowski(1) described the morphological and biological changes in the Ehrlich ascitic carcinoma of mice following infections induced with ceratin neurotropic viruses. These investigators have confirmed and extended these observations(2) with particular emphasis on the oncolytic action of the Bunyamwera virus after invasion of the malignant cells. This new experimental host-virus system has been employed by Ackerman (3) to propagate a neurotropic strain of Influenza A virus in serial passage. Price and Ginsberg(4) have demonstrated that infection of the ascitic tumor cells with Newcastle disease virus (NDV) resulted in a marked inhibition of growth of the malignant cells in the absence of demonstrable virus multiplication. In the course of their work with neurotropic viruses, Koprowska and Koprowski(2) showed that St. Louis encephalitis virus appeared in high titer in the ascitic fluid following infection although no consistent oncolytic effect was observed. The present communication reports the results of experiments dealing with the determination of the optimal conditions for the propagation of St. Louis encephalitis virus in the cells of the Ehrlich ascitic carcinoma, and the results of attempts to propagate the viral agent in these cells in serial passage. Some evidence bearing on the possible oncolytic action of SLE virus is presented.

Materials and methods. 1. *Virus.* The Webster strain of St. Louis encephalitis virus was employed in these experiments. Its LD_{50} titer approximated $10^{-7.0}$ when titrated in 3-4 week old mice by the intracerebral route of inoculation. 2. *Tissue.* The Ehrlich ascitic carcinoma(5) was obtained through the courtesy of Dr. J. C. Snyder. Prior to its use in these experiments it was carried through 15 serial mouse passages in our laboratory. For each passage ascitic fluid was removed from 3-6 mice inoculated 10-18 days previously. These fluids were pooled and diluted 10^{-2} in buffered saline or 0.2% bovine albumin broth. Each mouse received an intraperitoneal inoculum of 0.1 ml of this diluted ascitic fluid. The cell count of this inoculum (0.1 ml) approximated 100,000. 3. *Mice.* Adult Swiss white mice (Tumblebrook and CFW strains) were employed for the propagation of the ascitic tumor. Three 4 week old mice of the same strains were used in the virus titrations. No difference in the behavior of these 2 strains of mice has been noted. 4. *Determination of viral content of infected tumor cell suspensions.* Ascitic fluid was removed from at least 3 mice, pooled, quick frozen and stored in the CO_2 cabinet. After thawing, the ascitic fluid was centrifuged at 30,000 or 35,000 rpm at 4°C for 30 minutes. Since preliminary experiments had shown that the sedimented tumor cells contained approximately 10 times as much virus as the supernatant ascitic fluid, the latter was discarded. The sedimented tumor cells were washed 1-3 times in buffered saline or 0.2% bovine albumin broth and recentrifuged after each washing. After the final centrifugation the packed cells were diluted with 10 times their volume of 0.2% bovine albumin broth. The resulting suspension of tumor cells was quick-frozen and stored in the CO_2 cabinet until infectivity determinations could be carried out. At this time the tumor suspensions were thawed and after the addition of sterile alundum were ground by means of a chilled mortar and pestle. The resulting suspension was centrifuged at 8,000 rpm at 4°C for 15 minutes, and the supernatant fluid was drawn off and passed through a Seitz EK filter. The cell free filtrate was tested for infectivity by intracerebral inoculation of suitable 10-fold dilutions into 3-4 week old mice. The diluent employed was 0.2% bovine albumin broth.

---

* This investigation was supported in part by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army under Contract No. DA-49-007-MD-382.

TABLE I. LD_{50} Titer of SLE Virus.

Age of tumor at time of viral inoculation	Day after inoculation of SLE virus							
	1	2	4	6	8	10	12	14
0 (simultaneous)	<2.0*	3.3	4.5	4.5	<1.0	1.0	<1.0	—†
1	<1.0	<1.7	5.4	4.4	5.0	4.3	—	3.2
2	3.4	2.4	5.5	4.9	4.4	4.6	3.7	—
4	4.7	6.3	6.0	5.6	5.5	4.5	4.0	—
6	4.6	4.7	5.4	4.6	5.2	3.2	2.4	—
8	4.6	4.2	5.2	3.6	3.2	3.2	—	—
10	2.2	2.7	3.1	3.5	2.4	—	—	—
14	1.8	3.4	4.2	—	—	—	—	—

* Logarithm of reciprocal of LD_{50} dilution.
† Not tested.

and each mouse received an inoculum of 0.03 ml. Six mice were inoculated with each dilution and the LD_{50} titer was determined by the method of Reed and Muench(6). Surviving mice were observed for 12 days. 5. *Cytological technic.* Cell counts were carried out on various samples of ascitic fluid by means of a standard hemocytometer. One percent methylene blue was used as diluent. Only tumor cells were counted. Smears of viral infected and control ascitic tumor cells suspensions were prepared, fixed in formalin and stained with hematoxylin and eosin.

Plan of experiment and results. In the first experiment 200 adult mice were inoculated intraperitoneally with 0.1 ml of a 10^{-2} dilution of ascitic fluid and then divided into several groups. Mice of the first group received an immediate intraperitoneal injection of 0.1 ml of a 10^{-2} dilution of SLE virus. Other groups of mice were similarly injected with virus 1, 2, 4, 6, 8, 10 and 14 days after the inoculation of ascitic fluid, respectively. At regular intervals thereafter, mice from each group were sacrificed, the ascitic fluid removed and pooled, and the viral content of the centrifuged cells determined. In the event that no ascitic fluid could be obtained the peritoneal cavity was washed out with 3 ml of 0.2% bovine albumin broth. The results (Table I) show that in the case of mice inoculated simultaneously with tumor cells and virus an increase in the viral activity of the peritoneal washings or ascitic fluid could be demonstrated over a period of 4 days. Similar results were obtained in the case of mice inoculated with virus 1, 2, and 14 days after the implantation of tumor cells. Mice inoculated with virus 4, 6, 8 and 10 days after implantation of the tumor cells showed high titers 24 hours after inoculation, and no further significant increase could be demonstrated. Mice implanted with tumor cells 4 days before inoculation of a virus yielded the highest titers. As a general rule there was a decrease in viral titer after the 8th or 10th day following inoculation.

In the second experiment, 6 mice injected 6 days previously with the ascitic tumor were inoculated intraperitoneally with 0.1 ml of a 10^{-2} suspension of SLE virus which had an LD_{50} titer of approximately 10^{-7} when tested in mice by the intracerebral route of inoculation. Four days later all the mice were sacrificed and the ascitic fluid was removed. These fluids were pooled and an aliquot was removed and diluted to prepare a 10^{-3} suspension. Six mice inoculated 6 days previously with the tumor were injected with 0.1 ml of this diluted suspension. The remaining pooled fluid was ampuled and shell frozen for subsequent determination of infectivity titer. This procedure of continuous serial passage at 4 day intervals was repeated through 14 passages. The tumor cells of passages 1-5, 10 and 14, respectively, were washed, ground and filtered and the infectivity of the resulting filtrates determined. The results are summarized in Table II. Passages 1-5 revealed an LD_{50} titer of approximately $10^{-5.5}$. In passages 10 and 14 the infectivity titer had increased by one log to approximately $10^{-6.5}$.

In the third experiment, 35 adult mice were each injected with 0.1 ml of a 10^{-2} dilution of ascitic fluid. Four days later 17 of these mice received an intraperitoneal injection of

TABLE II. LD_{50} Titer of SLE Virus Propagated in Serial Passage.

Passage No.	LD_{50}
1	5.5*
2	5.3
3	5.7
4	5.6
5	5.7
10	6.6
14	6.6

* Logarithm of reciprocal of LD_{50} dilution.

a 10^{-2} dilution of SLE virus. The remaining 18 mice were left as controls. Both groups of mice were observed for signs of encephalitis and for the development of ascitic tumors. No deaths were observed which could be ascribed to viral infection. All mice of both groups gradually developed abdominal distention, and all died from the effect of the ascitic tumor. The mean survival time of the control group was 20 days after inoculation of the original ascitic fluids, as compared to 25 days in the group of mice injected with virus 4 days after the inoculation of tumor. This difference in mean survival time suggested the possibility that the virus might be exerting some oncolytic effect.

In the fourth experiment 23 adult mice were injected with 0.1 ml of a 10^{-2} dilution of ascitic fluid. Four days later 12 of the mice were injected intraperitoneally with 0.1 ml of a 10^{-2} dilution of 14th passage SLE infected ascitic fluid. The mean survival time of the two groups of mice was identical—viz.: 19 days after the inoculation of the original ascitic fluid. Five of the mice inoculated with 14th passage SLE infected ascitic fluid died with encephalitic signs within a period of 9-11 days after this inoculation. If these animals are eliminated the mean survival time of the 7 mice which died due to the effects of the tumor was 25 days, somewhat longer as compared to the control group. Eleven additional mice were injected with 0.1 ml of the 10^{-2} dilution of the 14th passage SLE infected ascitic fluid alone. Of these, 3 died with encephalitic signs presumably due to the effect of the virus on the 8th, 9th and 11th days after injection respectively; 3 gradually developed abdominal distention and ultimately died as a result of the tumor on the 25th, 25th and 47th day, respectively, while 5 survived throughout the 90-day observation period without developing any sign of tumor formation.

Although cell counts made on pools of ascitic fluid drawn from viral infected and control animals have shown consistently fewer cells in the former group the differences noted have not been dramatic. Furthermore, no significant differences have been noted between normal and viral infected tumor cells upon microscopic examination.

Discussion. The results of these experiments confirm the original observation of Koprowska and Koprowski(2) that the virus of St. Louis encephalitis multiplies in the cells of the Ehrlich mouse carcinoma. In addition, the SLE virus has been propagated without difficulty in serial passage in this ascitic tumor, and consistently high yields of virus have been obtained. Within broad limits the age of the tumor at the time of viral inoculation has not affected the final viral titer obtained to any significant degree although mice implanted with tumor cells 4 days before inoculation of the agent yielded the greatest amount of virus over the longest period of time (8-10 days after inoculation of the virus).

In the description of their experiments on the effect of certain neurotropic viruses upon Ehrlich mouse carcinoma Koprowska and Koprowski(2) described the results with SLE virus as equivocal in that a marked and consistent oncolytic effect could not be demonstrated although the virus was present in the ascitic fluid in high concentration. The results of the experiments reported in this paper are substantially in accord with these observations. Although no direct microscopic evidence of damage to the tumor cells as a result of viral infection was obtained, the fact that tumor bearing mice subsequently inoculated with virus tended to survive longer than control animals suggested that the virus was exerting some oncolytic effect. Additional evidence on this point was furnished by the lower tumor cell counts in viral infected animals, and by the fact that some mice inoculated with viral bearing tumor cells failed to develop ascitic tumors during the period of observation although all control animals did.†

The SLE virus appears to be one which parasitizes the tumor cells and proliferates readily while exerting no more than a slight oncolytic effect at best on the malignant cells in the process.

Summary. The virus of St. Louis encephalitis has been propagated in serial passage in the Ehrlich carcinoma of mice. The optimal conditions for the multiplication of the agent have been determined and relatively high viral titers have been obtained in the ascitic fluid. Some evidence suggesting a slight oncolytic effect has been obtained.

---

† Preliminary comparative Warburg studies carried out by Dr. Robert E. Olson on normal and viral infected tumor cells have shown no significant differences in endogenous metabolism, or in exogenous metabolism when the following substrates were added: pyruvate, lactate, succinate or glucose.

1. Koprowska, I., and Koprowski, H., *Fed. Proc.*, 1952, v11, 420.

2. ———, *J. Exp. Med.*, 1953, in press.

3. Ackerman, W., and Kurtz H., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 421.

4. Price, A., and Ginsberg, H., *Fed. Proc.*, 1953, v12, 455.

5. Klein, G., *Cancer*, 1950, v3, 1052.

6. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

Received July 2, 1953. P.S.E.B.M., 1953, v83

Consideration of Dose-Weight Relationships. (20505)

DAVID P. RALL AND WILLIAM C. NORTH. (Introduced by J. A. Wells.)

From the Department of Pharmacology, Northwestern University Medical School, Chicago, Ill.

Susceptibility of individual animals to the action of a drug is frequently expressed in terms of the dose required to produce a given effect. With some drugs this dose varies in a systematic fashion with the weight of the animal. It has become common practice to express dose as a linear function of body weight (mg/kg) in an effort to remove this source of systematic variation. That such a correction implies a direct proportionality between dose and weight of animal is not generally recognized, in spite of the fact that the inadequacies of this correction have been emphasized(1-5). Moreover, application of this correction to relationships other than a direct proportionality serves only to trade one type of systematic variation for another. Occasionally, this correction term has been applied when none was indicated, and erroneous interpretations of the influence of weight on susceptibility have resulted.

An example of the inappropriate use of the linear correction factor may be found in the susceptibility of wild Norway rats to alpha-naphthyl-thiourea (ANTU). Adequate data have been presented on this(6) to allow investigation of the dose/weight relationships.

On examining these data (Table I) it is apparent that the LD_{50} in mg/kg varies with weight, and in a systematic fashion. In the last column is found the LD_{50} in mg/animal for each weight group which we have calculated as the product of mean weight and LD_{50} in mg/kg. These values, in contrast to the LD_{50}'s in mg/kg, are relatively constant and show no systematic variation related to body weight. The mean LD_{50} is 2.63 mg/animal with a coefficient of variation of 15.6%. The comparable index of variation for the LD_{50}'s in mg/kg is 83.3%. The slope constant of the regression of LD_{50} in mg/animal on body

TABLE I.
Toxicity of ANTU for Wild Norway Rats.

No. rats*	Mean wt* (kg)	LD_{50} ± S.E.* (mg/kg)	LD_{50}† (mg/animal)
24	.039	58 ± 4.4	2.3
40	.081	43 ± 5.7	3.5
35	.112	22 ± 3.2	2.5
22	.140	18 ± 2.7	2.5
29	.170	16 ± .9	2.7
36	.263	8.1 ± .9	2.1
82	.348	7.7 ± 1.0	2.7
56	.448	6.2 ± .6	2.8

* From Dieke and Richter.

† Calculated as product of mean wt and LD_{50} in mg/kg.

weight is not significantly different from zero; and the line may be regarded as horizontal with the intercept (2.63 mg), representing an estimate of the LD_{50} in mg/animal for all weight groups. Thus, it is evident that the toxicity of ANTU for wild Norway rats is expressed with less variation as dose/animal than dose/unit weight of animal, and the use of the customary correction for body weight (mg/kg) in expressing the LD_{50} introduces systematic variation between weight groups.

These data, presenting only mean body weight and LD_{50} in mg/kg with its standard error, allow no estimation of the intra-group variation of the LD_{50} in mg/rat as obtained by calculation. It seemed of interest, therefore, to determine the resistance of rats of differing weight to ANTU and to administer the drug on a dose per animal basis. This permits estimation of variation within weight groups and also obviates any inaccuracies in converting an LD_{50} in mg/kg to LD_{50} in mg/animal.

Adult male Sprague-Dawley albino rats were placed according to body weight into 8 groups with a range of about 40 g in each group. The dose of ANTU, dissolved in 1 cc of propylene glycol, was injected intraperitoneally, 5 doses for each group. All animals not dead by 24 hours were classified as survivors. The LD_{50} in mg/animal and the 95% confidence limits were determined for each weight group by the method of Litchfield and Wilcoxon(7). In addition, the LD_{50}'s in mg/kg were obtained by the same method. The mean weight for each group was used to determine each of the 5 mg/kg doses.

Results. As may be seen from the data in Table II, the LD_{50}'s in mg/animal show no systematic variation and they all lie within the 95% confidence limits of each other. The mean value is 0.98 mg/rat and the coefficient of variation is 5%. The LD_{50} in mg/kg varies from 6.80-2.15, consistent with data presented in other reports(6,8), and coefficient of variation of these values is 60%. The slope constant of the regression of LD_{50} in mg/rat on body weight (0.00011) is not significantly different from zero, and again the regression line may be considered horizontal with the intercept (0.98) providing an estimation of the LD_{50}. Thus, for the albino, as well as the wild Norway rat, the dose of ANTU necessary to kill 50% of the animals is independent of body weight and may be properly expressed as mg ANTU/animal.

TABLE II. Toxicity of ANTU for Albino Rats.

No. rats	Mean wt (kg)	LD_{50} in mg/animal	LD_{50} in mg/kg
42	.154	1.02 (.79–1.31)*	6.80 (5.40–8.57)*
64	.207	.90 (.76–1.06)	4.33 (3.80–4.94)
123	.246	.91 (.74–1.12)	3.73 (3.11–4.48)
92	.282	.98 (.84–1.14)	3.50 (2.97–4.13)
52	.324	1.04 (.85–1.24)	3.48 (2.78–4.35)
49	.363	.99 (.79–1.25)	2.78 (2.06–3.75)
58	.407	1.00 (.86–1.16)	2.60 (2.18–3.09)
28	.443	.97 (.80–1.18)	2.15 (1.79–2.58)

* 95% confidence limits.

Discussion. The use of an improper weight correction factor has in this instance led to the erroneous conclusion that there is marked age and weight variation in the susceptibility of wild Norway rats to ANTU. Instead of removing body weight as a source of systematic variation, this correction has actually added it. It seems appropriate, therefore, to consider what constitutes a proper correction of dose for body weight. The first requirement for this correction is that it should minimize variation in effective dose between animals grouped according to weight as compared to variation within these weight groups. Further, variation in effective dose between weight groups must be random and not related to body weight. This obtains if there is no significant regression of corrected effective dose on body weight.

The correction factor may be determined from experimental data by deriving an equation that satisfactorily relates amount of drug necessary to produce a given effect to weight of animals. In general, if LD_{50}'s are plotted against mean weight of the animal groups, a smooth curve will be obtained. Since this relationship usually is curvilinear, rectification is necessary. For many drugs, the empirical procedure of plotting log dose against log weight will adequately rectify the data. This relationship may be expressed by the equation: $\text{dose} = (a)\ \text{weight}^{(b)}$. The value of the exponent (b) represents the slope of the line from the double log transformation, and (a) represents the intercept constant by which the dose is multiplied to remove the variation due to weight. The correction factor, therefore, is 1/a weight^b. There are numerous instances in which dose of drug is proportional to a power of body weight(2,3,9-11). If the value of (b) is unity, then a direct proportionality exists and the customary mg/kg correction is proper(12,13). If (b) is zero, then the correction factor becomes unity and dose is independent of body weight. This is illustrated by the data of ANTU in albino rats for which it can be shown that $Y = 0.98$ weight$^{0.03 \pm 0.06}$.

Summary. The toxicity of alpha-naphthyl thiourea for both wild pigmented and laboratory albino rats, when expressed as a dose per animal, appears to be constant over a wide age and weight range. The use of the customary mg/kg correction for body weight introduces marked systemic variation. The requirements for a proper correction factor of dose for weight of animal are considered and it is suggested that this factor should minimize variation in effective dose between groups of animals of different weights and make corrected dose independent of body weight.

1. Dreyer, G., and Walker, E. W. A., *Proc. Roy. Soc. Lond. Ser. B.*, 1914, v87, 319.

2. Clark, A. J., *Heffters, Handb. der exp. Pharmakol.*, 1937, v4, 165.

3. Dawson, W. T., *Ann. Int. Med.*, 1940, v13, 1594.

4. Gaddum, J. H., *Pharmacol. Rev.*, 1953, v5, 87.

5. Günther, B., and Odoriz, J. B., *J. Pharm. Exp. Therap.*, 1945, v83, 1

6. Dieke, S. K., and Richter, C. P., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 22.

7. Litchfield, J. T, Jr., and Wilcoxon, F., *J. Pharm. Exp. Therap.*, 1949, v96, 99.

8. Byerrum, R. U., and DuBois, K. P., *J. Pharm. Exp. Therap.*, 1947, v90, 321.

9. Bliss, C. I., *J. Exp. Biol.*, 1936, v13, 95.

10. Bliss, C. I., and Hanson, J. C., *J. Am. Pharm. Assn.*, 1939, v28, 521.

11. Laug, E. P., *J. Pharm. Exp. Therap.*, 1946, v86, 324.

12. Poe, C. F., Strong, J. G., and Witt, N. F., *J. Pharm. Exp. Therap.*, 1937, v61, 62.

13. Haag, H. B., and Corbell, R. L., Jr., *J. Pharm. Exp. Therap.*, 1940, v68, 45.

Received July 27, 1953. P.S.E.B.M., 1953, v83.

Effect of Phenylbutazone (Butazolidin) on Renal Excretion of P-Aminohippurate and Thiosulfate in the Dog.[*] (20506)

MARY ELIZABETH KING.[†] (Introduced by A. Gilman.)

From the Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York City.

This study of the effect of phenylbutazone[‡] on the renal tubular transport of p-aminohippurate (PAH) and thiosulfate was prompted by a report that the drug produced a striking diminution in the serum uric acid of patients with gout(1). It seemed possible, therefore, that the drug might be a uricosuric agent. If so, it would be of interest to determine whether it resembles p-(di-n-propylsulfamyl)-benzoic acid (Benemid), which blocks PAH but not thiosulfate transport, or 4'Carboxyphenylmethanesulfonanilide (Caronamide) which blocks both PAH and thiosulfate(2). Subsequent reports as to the uricosuric activity of phenylbutazone are conflicting. One group of investigators states that there is "no marked increase in urinary excretion of urates"(3) while another believes that the drug "usually increases urinary excretion of urates"(4). Phenylbutazone has also been said to inhibit

---

[*] This work was supported by a research grant from the National Institute of Health, Public Health Service.

[†] Public Health Research Fellow of the National Institute of Health.

Present address: Army Medical Research Laboratory, Fort Knox, Ky.

[‡] Butazolidin was generously supplied by the Masonic Foundation for Medical Research and Human Welfare.

TABLE I. Effect of Butazone and Sodium Acetate on Tm PAH and T thio.

Exp.	Sex	Drug*	Clearance period	C_{Cr}	C_{thio}/C_{Cr}	T_{thio}† (mg/min.)	C_{PAH}/C_{Cr}‡	Tm PAH (mg/min.)
A	♂	None	1, 2	26	1.17	1.6	1.42	7.0
		Butazone	3	11	1.28	1.2	.98	.5
		"	4	18	1.38	2.7	1.09	2.2
B	♀	None	1, 2	46			1.45	5.8
		Butazone	3, 4	42			1.07	1.8
		Butazone & acetate	5, 6	43			1.18	5.9

* Prime: 50 mg/kg. Sustain: 50 mg/kg/hr.

† T_{thio} (tubular transport) is used rather than Tm because tubular resorption may occur concomitantly with secretion. Therefore, T_{thio} is not necessarily a maximum.

‡ PAH considered 92% filterable.

the excretion of p-aminosalicylic acid(5).

It has previously been shown that a rapid infusion of isotonic acetate increases the renal secretion of PAH(6) and is capable of reversing the inhibition of tubular transport caused by Caronamide(7). It was found in this study that phenylbutazone did block PAH transport; therefore, the effect of Na acetate on this inhibition was investigated.

Methods. All experiments were performed on anaesthetized dogs, 5 males and 3 females. Male dogs were chosen because of their ability to secrete much larger amounts of thiosulfate than normal females. The dogs were fasted for 18 hours. During this period, water was allowed as desired. After the intravenous injection of an initial priming dose of creatinine, thiosulfate and PAH, these substances were continuously infused at a rate appropriate for the maintenance of the desired serum levels. The level was around 20-30 mg/100 cc in the case of thiosulfate and creatinine and 50 mg/100 cc for PAH. Clearance periods of 20 minutes were used after an initial equilibration time of 30 minutes. Urine was collected by means of an indwelling catheter and the bladder flushed with 15 cc of distilled water at the end of each period. Blood samples were drawn from the jugular vein at the midpoint of each clearance period without correction for kidney to bladder delay. After 2 control periods, a priming dose of Phenylbutazone (50 mg/kg) was given intravenously and subsequently the drug was incorporated into the sustaining infusion (50 mg/kg/hr). Na acetate was given as an isotonic solution at the rate of 10 cc/min. Creatinine was determined by the method of Folin and Wu(8); PAH by the method of Smith *et al.*(9); and thiosulfate by the method of Gilman, Philips, and Koelle (10) with the modification of Elliott and Scott (11) for analysis in the presence of PAH.

Results and discussion. The initial priming dose of phenylbutazone produced a clear-cut fall in the ratio C_{PAH}/C_{Cr} in all the dogs studied. In 6 experiments the clearance ratios of the control period as compared to that of the experimental period fell as follows: 1) 1.52 to 1.06 2) 1.28 to 0.88 3) 1.49 to 0.94 4) 1.42 to 0.98 5) 1.45 to 1.07 and 6) 1.86 to 1.13. Occasionally there was a small rise in TmPAH (averaging less than 2 mg/min) during the second period after the priming solution was given. This occurred despite the sustaining dose of drug. A representative experiment is shown in Table I, Exp. A. The data indicate that phenylbutazone inhibits the tubular transport of PAH.

Secretion of thiosulfate in excess of filtration and concomitant resorption continued unchanged or slightly increased. Thus, in dogs, phenylbutazone blocks the secretion of the organic acid, PAH, but not that of the inorganic anion, thiosulfate. In this, it resembles Benemid as well as 2,4 dinitrophenol and dehydroacetate(2). Unlike Caronamide, the drug does not affect thiosulfate secretion.

In 2 experiments a rapid infusion (10 cc/min) of approximately physiological concentrations of $NaHCO_3$ and NaCl was given throughout the control and first 2 phenylbutazone periods. Following this, isotonic Na acetate was substituted for 2 periods. In one dog, the initial TmPAH averaged 15.5 mg/min and dropped to 6.4 mg/min after phenylbutazone. During the simultaneous infusion of acetate (4 mEq/kg/hr), the TmPAH rose from 6.4 mg/min to 12.3 mg/min. In another animal, the control Tm was 5.8 mg/min. It dropped to 1.8 mg/min after phenylbutazone was given and rose to 5.9 mg/min after the administration of Na acetate (8.9 mEq/kg/hr). (See Table I, Exp. B). Thus, it would seem that the rapid intravenous infusion of Na acetate will partially or wholly reverse phenylbutazone inhibition of TmPAH as measured by clearance studies. It has previously been shown to reverse Caronamide inhibition.

In the first experiment, intravenous phenylbutazone (200 mg/kg) caused marked hyperventilation, hemolysis, coma and terminal bloody diarrhea. A subsequent reduction in dose to 50 mg/kg in the priming solution and 50 mg/kg/hr in the sustaining solution was well tolerated in 2 experiments. In 2 other dogs, this dose caused hyperventilation followed by coma. Autopsy of 2 dogs was unrevealing as to the cause of death. The remaining animals were sacrificed immediately after the experiment.

Summary. Phenylbutazone blocks PAH transport but does not affect thiosulfate excretion. The inhibition of TmPAH by phenylbutazone is reversible by the simultaneous infusion of isotonic Na acetate.

The author is indebted to Dr. Alfred Gilman for his helpful criticism and the use of his laboratory facilities.

1. Kuzell, W. C., Schaffarzick, R. W., Brown, B., and Mankle, E. A., *J.A.M.A.*, 1952, v149, 729.

2. Foulks, J., Brazeau, P., Koelle, E. S, and Gilman, A., *Am. J. Physiol.*, 1952, v168, 77.

3. Kuzell, W. C., and Schaffarzick, R. W., *Bull. Rheum. Dis.*, 1952, v3, 23.

4. Gutman, A. B., and Yu, T. F., *Am. J. Med.*, 1952, v13, 744.

5. Pulver, R., and Wilhelmi, G., *Schweiz. Tuberk.*, 1952, v9, 86.

6. Mudge, G. H., and Taggart, J. V., *Am. J. Physiol.*, 1950, v161, 191.

7. Shideman, F. E., and Rene, R. M., *J. Pharm. and Exp. Therap.*, 1951, v101, 32.

8. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, v38, 81.

9. Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M., *J. Clin. Invest.*, 1945, v24, 388.

10. Gilman, A., Philips, F. S., and Koelle, E. S., *Am. J. Physiol.*, 1946, v146, 348.

11. Elliott, S. R., and Scott, H. W., Jr., *Bull. Johns Hopkins Hosp.*, 1948, v83, 213.

Received July 22, 1953. P.S.E.B.M., 1953, v83.

Mosaic Photography in the Demonstration of Human Synapses (*Boutons terminaux*).* (20507)

WILLIAM C. GIBSON AND V. A. PURKIS. (Introduced by D. H. Copp.)

From Crease Clinic Research Unit and Department of Neurological Research, University of British Columbia, Vancouver, B. C., Canada.

The objective demonstration of human cerebral synapses has long been thought to be of considerable interest and importance. However, at the high magnifications required (approximately 2000 x) it has been difficult, in one photomicrograph, to show a convincing number of synapses. As a result, drawings have usually been resorted to, and photographic proofs of the size and distribution of the *Boutons terminaux* have been wanting.

The branching nature of nerve cells has also served to make difficult the picturing, in any single photograph, of the synapses on the dendrites. In many instances the number and location of synapses on the dendrites may be more important that on the "cell body," narrowly interpreted.

The thickness of frozen section of nervous tissue for silver staining (approximately 15 μ) allows one to follow the dendrites great distances, at times. Thinner sections are not very practical since metallic stains make them brittle and since nerve cell processes

---

* This research supported by a Federal Mental Health Grant.

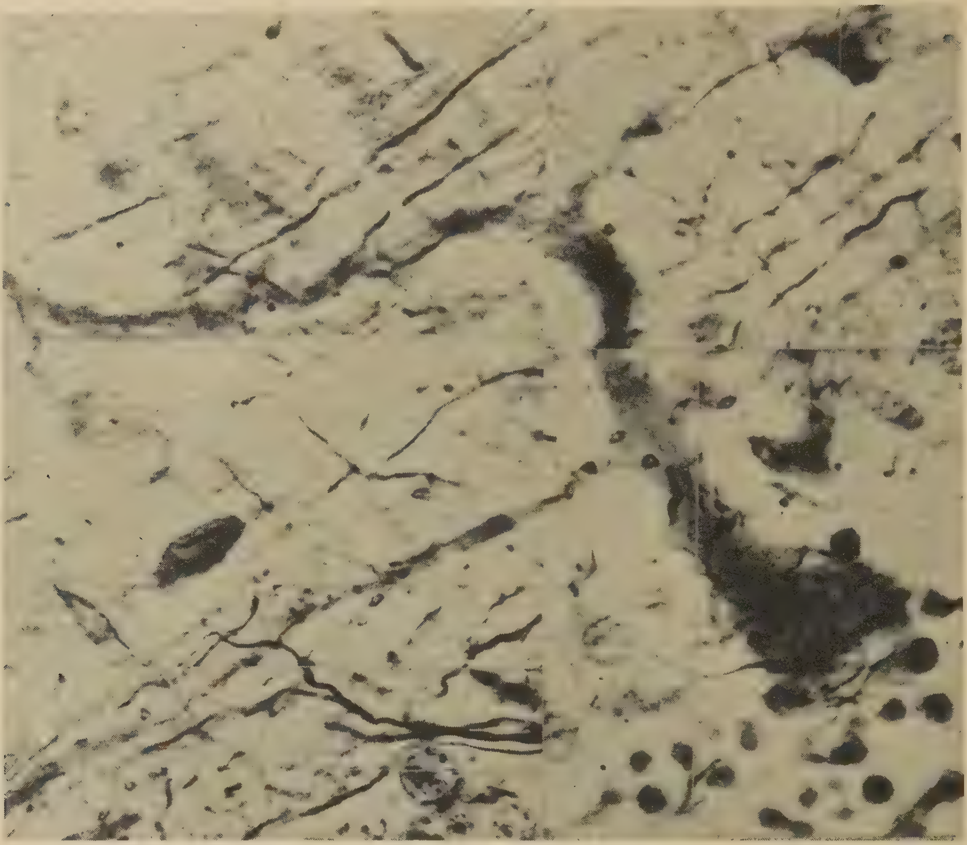


FIG. 1. Synapses on Purkinje cell, normal human cerebellum. ×2000.

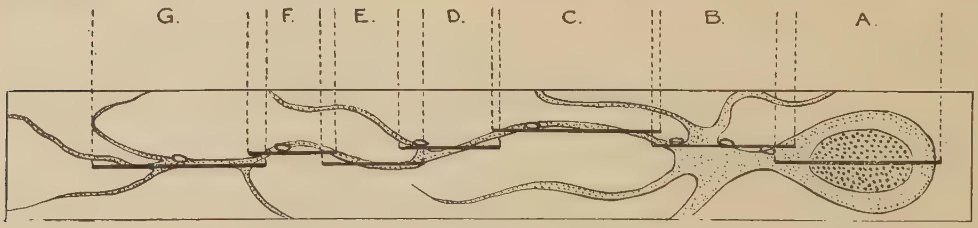


FIG. 2. Cross-sectional sketch of hypothetical cell to show levels of focus required to make up a complete photo-mosaic. Section A of mosaic is taken at level indicated to gain maximal cell diameter, in order that branching dendrites in Section B may be joined in proper perspective. Section E illustrates the fact that in order to preserve continuity of dendrite, photographs must occasionally be taken where synapses are not completely visible.

are sacrificed. For these reasons it was felt that overlap aerial photographic technics might be applied to the synaptic problem. In simplest form, the method is as follows: Each terminal bouton is photographed in focus, a considerable overlap being allowed on either side of it. Occasionally more than one axon terminal can be included in the one focal plane, but for the best results it is usually necessary to treat each bouton ending separately. If a good margin of overlap has been allowed, and if certain desirable landmarks have been consistently included, (capillaries, large thick fibres or cell borders) it is not difficult to assemble the essential parts of each print into a mosaic. In the illustration given here (Fig. 1) 9 separate negatives were used to record the *Boutons terminaux* found at 9 focal planes of a Purkinje cell of a normal human cerebellum stained by Rio-Hortega double impregnation silver method(1). In this mosaic it has been possible to demonstrate 16 boutons, while a single film would have shown 4 at most. All the processes of the Purkinje cells existing in the 15 μ section could have been followed out by this method if it had been required.

Summary. One solution to the problem of the photomicrographic demonstration of *Boutons terminaux* in the normal human brain is proposed in the form of in-focus mosaic photography.

---

1. Morrison, G. E., and Gibson, W. C., *Science*, 1953, v117, 656.

Received July 3, 1953. P.S.E.B.M., 1953, v83.

The Tetracaine Flare: Differences in Actions of Procaine and Tetracaine on Peripheral Nerve.*† (20508)

R. R. Sonnenschein.

From the Department of Physiology, University of California School of Medicine, Los Angeles.

It is well known that trauma of the skin or the intradermal injection of histamine results in the development of a flare, an area of erythema extending from the site of injection for several centimeters. The studies of Lewis(1,2) and others have established that the flare is brought about by a local nervous mechanism, the so-called axon reflex. In support of this concept is the observation which we have repeatedly confirmed(3,4) that previous infiltration of the skin with the local anesthetic procaine, in the area where histamine is to be injected, prevents the development of the flare, although the local wheal produced by histamine is not affected.

Our attention was drawn(5) to the paradoxical observation that tetracaine, a potent local anesthetic related chemically to procaine, itself produces a flare when injected intradermally even while causing gross anesthesia. This phenomenon appeared to offer the opportunity for testing the concept of the axon reflex as the basis of the flare and for further elucidating its mechanism. The present communication deals with this problem and offers the results of our attempts at its solution.

Material and methods. In all cases, injections of the various agents were made intradermally with 26 or 27-gauge needles in measured volumes from tuberculin syringes. Sterile crystals of procaine hydrochloride‡ and tetracaine hydrochloride‡ were dissolved in sterile isotonic saline to the desired concentrations, expressed as weight/volume. When either agent was injected alone, the volume was 0.1 ml. When the blocking action of procaine on the flare of tetracaine was tested, the former was first infiltrated into the skin in a volume of 0.2 ml, and a few seconds later 0.05 ml of tetracaine solution was injected into the center of the anesthetized area. In all experiments a control was obtained for the dilution factor by injecting the same volume of tetracaine into 0.2 ml of isotonic saline.

To determine the effects of procaine and tetracaine on pain thresholds, the drugs were injected in volumes of 0.1 ml into the skin of the volar surface of the wrist. The thresholds for "fast" (immediate) and "slow" (delayed) pain were determined by use of the warm-wire algesimeter of Lee, Williams and Pfeiffer(6). A bent resistance wire, heated by a battery to various temperatures indirectly indicated by an ammeter, was lightly and briefly applied to the skin. At sub-threshold temperatures only touch was felt. When the threshold for slow pain was reached, the pain was felt after an interval of about one second following the touch. Further increase in temperature finally resulted in elicitation of fast pain occurring simultaneously with the

---

* Supported by a Lilly Research Grant.

† Preliminary experiments were done in collaboration with Dr. Henry D. Janowitz, Mount Sinai Hospital, N. Y. City.

‡ Procaine (Novocaine) and tetracaine (Pontocaine) were generously supplied by Mr. Harold Epley, Winthrop-Stearns, Inc.

touch. When fast pain occurred it was usually no longer possible to discriminate the slow pain. Since the threshold for fast pain was always 30 to 50% higher than that for slow pain, however, it was assumed that whenever fast pain occurred the stimulus was above threshold for slow pain. When control determinations were being made the wire was touched to 5 different spots for each temperature. The threshold for each type of pain was taken as that ammeter reading at which the wire consistently produced pain in 2 or 3 of the 5 trials. After the anesthetics were injected, the thresholds were determined every 15 to 30 seconds until they returned to the control level. Since the thresholds changed rapidly, each test at a given temperature consisted of only two applications of the wire rather than 5, and if one of the 2 resulted in a positive response, that stimulus was considered to be at or above thresholds at that moment. In all cases, the thresholds for both types of pain were repeatedly checked during the experiment. The reported times for full recovery are accurate only to within 1 to 3 minutes. The concentrations were selected so as to give an effect lasting about 10 to 20 minutes.

Statistical significance of the results of the last experiment was determined in two ways. First, the mean differences between times for recovery of slow pain and fast pain (S-F) under procaine was compared with the mean difference under tetracaine by use of Fisher's t-test(7a). Secondly, the adjusted chi-square for a 4-fold table(7b) was determined where the two classes of the one attribute were *a* duration of effect on slow pain greater than on fast pain ($S > F$), and *b* ($S \leq F$); and the two classes of the second attribute were *a* procaine and *b* tetracaine.

Results. A. *The flare and its blocking by procaine.* Thirty-three tests were made with tetracaine at concentrations from 1% to 0.0001%. The threshold for production of flare appeared to be about 0.001%, since at this level 2 of 6 tests gave a positive response, while none of 4 at 0.0001% did so. At 0.01%, 4 out of 5 were positive; higher concentrations uniformly caused the flare. In other respects, excepting the local anesthesia, the response to tetracaine was identical with that of histamine: the local wheal, the surrounding hyperalgesia and "itchy skin" were all observed. Injections of 1% tetracaine caused definite irritation of the skin and in two cases a local necrosis and slough ensued. The flare produced by 0.1% tetracaine was roughly of the same magnitude as that of 0.001% histamine. Redness appeared within seconds of the injection, gradually increasing in extent and intensity to cover a roughly circular area of 2 to 5 cm diameter.

Ten tests were made to determine the effect of procaine on the tetracaine flare. In 2 cases, 1% procaine was shown to block completely the effect of 1% tetracaine; in 7 of 8 experiments 0.2% procaine blocked 0.2% tetracaine. No further attempt was made to establish dosage relationships between the two agents. Procaine itself produced only a local reddening at the site of injection, presumably due to its anesthetization of local vasoconstrictor fibers. B. *Effects of tetracaine and procaine on conduction in isolated nerve. Preliminary experiments.* Thus, it appeared that tetracaine, while producing a marked local anesthesia, can evoke a neurally mediated flare, and that procaine is able to block the flare. These facts suggested 1) that the fibers involved in the axon reflex for flare may be different from those which participate in the gross sensory response; and 2) that tetracaine and procaine act differently on the two groups of fibers, the former failing to block completely the one group, thus allowing the flare to develop, the latter effectively blocking all the fibers concerned. To test the latter suggestion, the effects of the two agents on conduction in the isolated, desheathed bull frog sciatic nerve were investigated.§ The results may be summarized as follows: 1. Tetracaine had an overall potency about 100 times that of procaine on a molar basis. 2. On washing away the anesthetic from a nerve whose conduction had been completely blocked, in the case of procaine (as is reported for cocaine (8)) the A fibers (rapidly conducting) recov-

---

§ These experiments were kindly performed by Dr. F. S. Crescitelli. Future definitive experiments are planned.

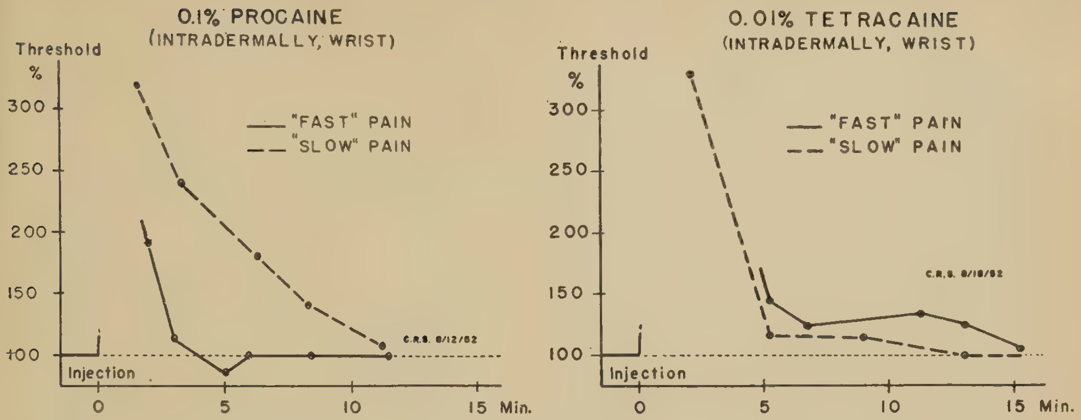


FIG. 1. a (left). Responses of slow and fast pain thresholds in a subject after injection of procaine. b (right). Ditto, same subject after tetracaine.

ered much sooner than did the C fibers (slowly conducting). The converse occurred with tetracaine in that recovery of the C fibers was more rapid than that of the A fibers. In the initial phase of blocking after application of the anesthetic to the nerve, corresponding effects were observed but the differences were not as marked: with procaine, the C fibers were blocked more rapidly than the A fibers; with tetracaine the opposite occurred.

These observations lent support to the suggestion that the two agents have different effects on conduction among the different groups of fibers, tetracaine manifesting much less blocking action on C fibers with respect to A fibers than is the case with procaine.

C. *Effects of tetracaine and procaine on thresholds of "fast" and "slow" pain.* The preliminary observations on isolated nerve, showing differences in action between the 2 local anesthetics, suggested a corollary experiment in the human skin. The "double response" of pain(9) presented itself as an ideal situation for testing function of A and C fibers. It has been inferred(9) that "fast" or immediate pain is mediated over rapidly conducting fibers probably of the A group, while "slow" or delayed pain is mediated over slowly conducting fibers of the C group. By determining alterations in thresholds of the two responses one may secure an indication of the functional status of the two groups of fibers.

The effects of tetracaine and procaine on thresholds of fast and slow pain were determined on 6 adult, white, male subjects in the manner described above. The results of a representative experiment with procaine are shown in Fig. 1a, and its pair with tetracaine in Fig. 1b. It is apparent that under procaine recovery of fast pain occurred much sooner than that of slow pain. Conversely, under tetracaine, recovery of slow pain took place much earlier with respect to fast pain. In other words, tetracaine had relatively much less effect on fibers mediating slow pain with respect to its action on fast pain than was the case with procaine. Fig. 2 presents the results of all experiments and shows that the same trend was present throughout with

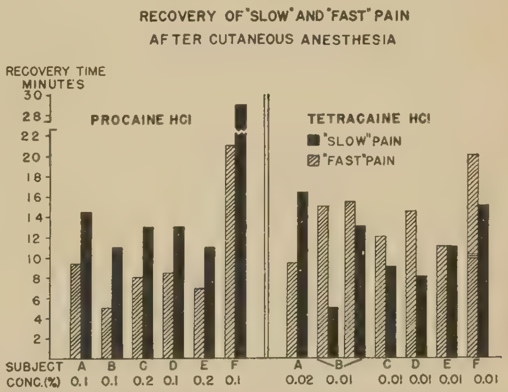


FIG. 2. Results of all experiments on the effects of procaine and tetracaine on slow and fast pain. In the experiment on subject F with tetracaine, there was apparent initial recovery of fast pain at 10 min.; threshold again rose and did not recover until 20 min.

the exception of one test with tetracaine. These differences between the 2 agents are statistically significant to within the 5% confidence limit when calculated by either of 2 methods described above. Determinations of thresholds under tetracaine were more difficult than under procaine, because of the itching produced by tetracaine. This may have contributed towards the lesser regularity of the tetracaine results.

Discussion. The results obtained suggest a reasonable explanation for the paradoxical tetracaine flare. First, as previously recognized(10,11), tetracaine is an irritant to tissue and thus satisfies a requisite for initiation of the flare. Whether tetracaine releases histamine locally or whether it may be intrinsically histamine-like is unknown. When the agent is injected intradermally, the central portion of the injection site may be presumed to have the highest concentration, so that gross testing at this point may reveal clinically complete anesthesia. Toward the periphery of the injection wheal, however, particularly as lateral diffusion takes place, the effective concentration falls off. Thus, at the periphery, the concentration may be high enough to initiate the flare and yet not be high enough to block conduction in the fibers which mediate the response.

The facts here demonstrated, that tetracaine has a lesser effect than procaine on C fibers and that procaine blocks the flare, strongly suggest that the flare is mediated over C fibers. The findings throw no light on the question of further identity of the flare fibers, however. Granting that they are probably of the C group, the two alternatives remain: that they are of the known afferent group of posterior root fibers, or that they are of a separate system, the "nocifensor nerves" of Lewis. The present study offers no evidence for a selection between these alternatives. It is tempting to speculate on the relationship of the flare fibers to the so-called posterior-root vasodilators.

Finally, the difference in effects of procaine and tetracaine on conduction in different groups of nerve fibers is in itself of interest. That members of this class of local anesthetics may differ in this respect has not been suspected, to our knowledge. The basis for the differences is entirely speculative; a possibility that comes to mind is that a difference in rates of diffusion to cell membranes may exist. Everett and Goodsell(12) reported that procaine may spare a group of C fibers when conduction in isolated nerve is tested. This is reminiscent of our results with tetracaine. The discrepancy with our findings on procaine is at present unaccountable.

Summary. 1. Tetracaine hydrochloride, injected intradermally, produces a histamine-like flare which can be blocked by procaine hydrochloride. 2. On the isolated nerve, tetracaine has much less effect on conduction in C fibers with respect to its effect on A fibers than is the case with procaine. (Preliminary experiments.) 3. In the human skin, tetracaine has much less effect on the threshold for "slow" pain with respect to its effect on "fast" pain than is the case with procaine. 4. These observations suggest that the flare is mediated over C fibers. Tetracaine produces a flare 1) by causing irritation, 2) by failing to block completely the C fibers. 5. The differences in action on conduction of tetracaine and procaine are noted, but no explanation for these differences is available.

---

1. Lewis, T., and Grant, R. T., *Heart,* 1924, v11, 209.

2. Lewis, T., Grant, R. T., and Harris, K. E., *Heart,* 1927, v14, 1.

3. Janowitz, H. D., and Sonnenschein, R. R., unpublished observations.

4. Bernstein, M., and Sonnenschein, R. R., unpublished observations.

5. Pfeiffer, C. C., personal communication.

6. Lee, R. E., Williams, H. L., and Pfeiffer, C. C., *Fed. Proc.,* 1949, v8, 314.

7. a. Snedecor, G. W., *Statistical Methods,* Ames, Iowa State College Press, 4th Ed., 1946, 75-78.

b. *ibid.,* 194-197.

8. Gasser, H. S., and Erlanger, J., *Am. J. Physiol.,* 1929, v88, 581.

9. Lewis, T., *Pain,* New York, The Macmillan Co., 1942.

10. Dietrich, W. C., and Beutner, R., *Anesthesiology,* 1946, v7, 255.

11. Hamilton, H. S., Westfall, B. A., and Ferguson, J. K. W., *J. Pharm. and Exp. Therap.,* 1948, v94, 299.

12. Everett, G. M., and Goodsell, J. S., *J. Pharm. and Exp. Therap.,* 1952, v106, 385.

Received July 27, 1953. P.S.E.B.M., 1953, v83.

Experimental Production of Struma Lymphomatosa.* (20509)

H. J. Clausen.

From the Department of Anatomy, University of South Dakota School of Medicine, Vermillion.

In a series of experimental investigations dealing with the effects of the prolonged administration of thiouracil to rats(1), it was noted that some of the animals developed a thyroid condition similar to that which has often been reported under the general heading of chronic thyroiditis in man(2-5).

As far as the author has been able to determine, only one report(6) of the experimental production of chronic degenerative thyroiditis, namely struma lymphomatosa (Hashimoto), has been reported to date. In view of this, it was thought of sufficient importance to report the following findings.

Material and methods. A series of 21-day-old Sprague-Dawley rats were given a .1% thiouracil† solution as a substitute for their drinking water. The food consisted of Purina laboratory chow. Littermate control animals were carried along with the above experimental rats on a similar diet without the thiouracil in their drinking water. All animals in both groups were sacrificed 600 days following the initiation of the experiment. Various tissues, including the thyroid glands, were removed at this time. Tissues were fixed in formalin, Bouin's or Zenker's fixative. The thyroid glands were stained with hematoxylin and eosin for the most part. Some sections were stained with Mallory's connective tissue stains.

Results. Adenomatous formations of various types were found in the thyroid glands of the majority of the experimental animals. Various changes in the parathyroid glands were also noted. The above findings are being further investigated and will be reported at a later date.

In 6 of the animals within the experimental series (25%), the thyroid glands were found to exhibit a characteristic chronic thyroiditis of the Hashimoto type. Upon removal of each of the thyroid glands, prior to their fixation, it was noted that the gland was very much increased in size. The tissue was white and very firm and little or no lobulation was in evidence. No cysts were recognized grossly.

Microscopically, it was apparent that the capsule was increased in thickness. When compared to the controls, it could be noted that there was a diffuse and uniform degeneration of the acini with a general flattening of the epithelium and an absence of colloid in the treated glands (Fig. 1, 2). The epithelial elements were reduced in size and many eccentrically placed nuclei were present. Extensive interlobular and intralobular lymphocytic infiltration was an outstanding characteristic in most of the cases. A consistent increase in fibrous tissue (Fig. 3) surrounding the lobules of degenerating acini was generally noted. The small arteries and the precapillary arterioles uniformly showed a definite thickening of the intima and the media. In many of the lobules the epithelium appeared to be replaced by a rather dense fibrous tissue. Mitotic figures or other indications of hyperactive epithelial proliferation was not observed.

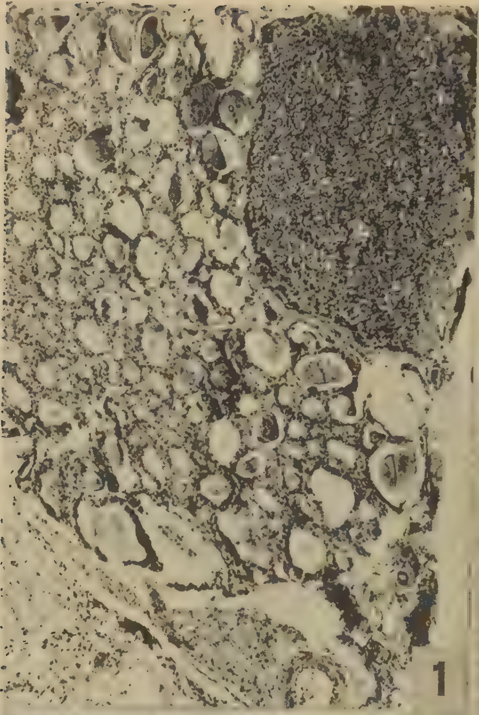
In each of the rats in which the above thyroid findings could be demonstrated, the testes were also found to be undergoing abnormal or pathological changes, (Fig. 4). The testes were all normal in size as compared to the normal littermate controls. The tunica was increased in thickness with a very dense fibrous tissue. It was noted that most of the tubules were filled with immature and degenerating germ cells. No evidence of normal mature sperm was found. In some areas some inter and intratubular lymphocytic infiltration could be seen. The interstitial cells appeared to be normal in most areas of the testis.

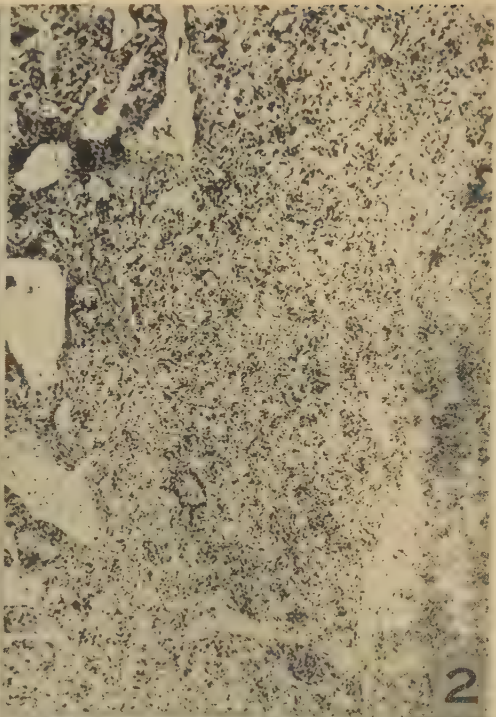
The adrenal glands were smaller in size than those of the normal controls but all of the cellular elements appeared to be normal in structure and relative proportions. The pituitary glands of all of the rats in the series are now being studied and will be reported.

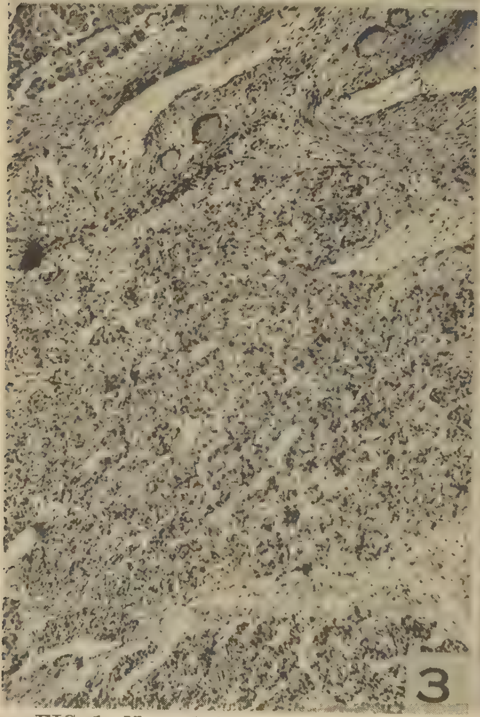
---

* Aided by a research grant from the South Dakota Division of the American Cancer Society

† Furnished through the courtesy of the Lederle Laboratories Division, American Cyanamid Co., New York City.







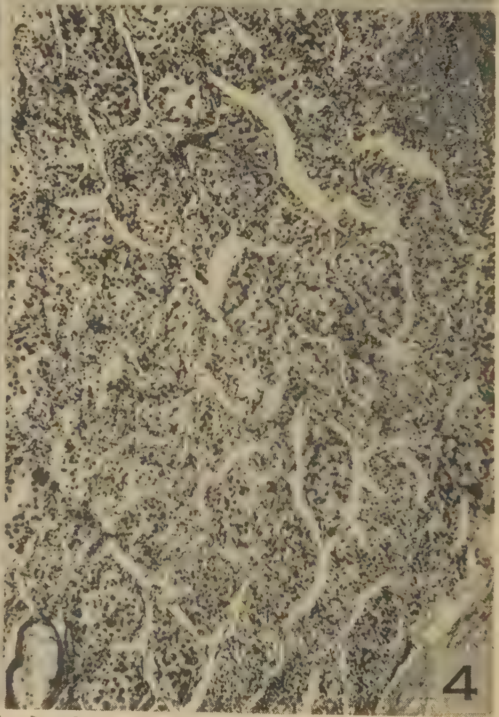


FIG. 1. Normal thyroid and parathyroid glands of a rat. A littermate control. (H & E) ×100.

FIG. 2. Thyroid gland after thiouracil administration. Fibrolymphoid hyperplasia with lymphocytic infiltration involving the intervesicular connective tissue. A portion of the parathyroid is shown in the upper left corner. (H & E) ×100.

FIG. 3. Thyroid of another experimental rat (K3). More advanced stage of fibrolymphoid hyperplasia. The widespread connective tissue deposition and almost complete absence of any follicular structures may be noted. (H & E) ×100.

FIG. 4. Testis of experimental rat (K3). Lymphocytic infiltration may be noted in some of the intertubular areas. Tubules appear to be filled with immature and degenerating germ cells. (H & E) ×100.

Discussion. Inflammation in which the thyroid tissue was to a large extent replaced by lymphoid tissue was described by Hashimoto (7). Microscopically, the lymphoid tissue in the 4 human cases he reported was so abundant that he chose the name "struma lymphomatosa" for the lesion.

The etiology of struma lymphomatosa is unknown. Infections of various types have been thought at times to be the causative factors. Cultures of glands, whenever carried out, have been sterile and no cases could be produced when bacteria were injected directly into the thyroid arteries of dogs and rabbits(5). Furthermore, if infection were the causative factor it is unlikely that our littermate control rats would have entirely escaped the infection. It is entirely possible that infection might have played an indirect role. The administration of thiouracil for such a prolonged period might have lowered the metabolic activity and thus decreased the resistance of the animals involved.

That iodine ingestion is a possible factor in the production of this characteristic thyroid picture has been suggested(8). However, in many of the human cases reported no history of iodine ingestion was present. The frequency of iodine administration to patients having thyroid disease of any sort and the relative rarity of struma lymphomatosa should tend to eliminate iodine as the etiological agent concerned.

Certain dietary deficiencies have been considered and McCarrison(6) has produced lesions in rats which showed a definite microscopic resemblance to struma lymphomatosa. He obtained this thyroid condition in the same percentage of cases in the series as the author is reporting. The above investigator has suggested that a vitamin-deficient diet was the possible etiological factor. It is possible that some goitrogenic substance may have been present in the diet that the above investigator used. No dietary insufficiency appeared to be in evidence in either our control or experimental rats. Furthermore, in many human cases of this disease no history of any dietary inadequacies has been reported.

Testicular changes similar to those obtained in our experimental rats have not been reported in any of the human cases of Hashimoto's disease so far as the author was able to determine. However, since this disease occurs chiefly in females and also in the older age groups of either sex it is possible that these changes may not have been severe enough to have been reported for the human. While numerous reports on the thyroid-sex gland relationships are somewhat contradictory(9), our results would tend to suggest that the hypothyroid condition which must exist in these cases had no influence on the interstitial cells of the testis. The results obtained in relation to the germ cells might have been caused by the direct irritation of the chemical used or it might be mediated through thyroid-pituitary secretions. Further studies in this regard are now being made.

Summary. 1. The effects of prolonged administration of thiouracil to male rats was studied. Following the drug administration for a 600-day period, 25% of the rats showed thyroid glands which exhibited histological characteristics similar to that found in struma lymphomatosa. 2. The testes of these animals also showed a failure of germ cell development and maturation as well as some lymphoid infiltration.

---

1. Clausen, H. J., *Anat. Rec.*, 1951, v111, (suppl. 3) 80.

2. Riedel, B. M. C. L., *Verhandl. d. deutsch. Gesellsch. f. Chir.*, 1897, v26, 127.

3. McClintock, J. C., and Wright, A. W., *Ann. Surg.*, 1937, v106, 11.

4. Joll, C. A., *Brit. J. Surg.*, 1939, v27, 351.

5. Womack, N. A., *Surgery*, 1944, v16, 770.

6. McCarrison, R., *Brit. Med. J.*, 1929, v1, 5.

7. Hashimoto, H., *Arch. Klin. Chir.*, 1912, v97, 219.

8. Boyden, A. M., Coller, F. A., and Bugher, J. C., *West. J. Surg.*, 1935, v43, 547.

9. Crotti, A., *Thyroid and Thymus*, Lea & Febiger, Philadelphia, 1938.

Received July 3, 1953. P.S.E.B.M., 1953, v83.

Histochemical Studies on the Clam *Mactra solidissima*. (20510)

Robert Love and Laurence H. Frommhagen. (Introduced by Thomas H. Jukes.)
From Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

For many years it has been known that certain species of molluscs are rich in acid mucopolysaccharide(1-6). The presence of a heparin-like factor in clams was shown by Thomas (7). Subsequently, Frommhagen *et al.*(8) isolated acid mucopolysaccharides from 2 species of clams and characterized them as sulfated polysaccharides similar to heparin and having a strong heparin-like activity in preventing the coagulation of blood.* In view of the supposed relationship between mast cells and heparin formation the following study was undertaken primarily to investigate the localization of mucopolysaccharide in the clam.

Materials and methods. Mactra solidissima was used for these experiments since this is the clam from which the anticoagulant substance (mactin-A) is routinely extracted in this laboratory.

Histochemical methods. Living clams were dissected and blocks of the mantle, syphons, palps, gills, foot, muscles, and body were fixed in 10% formaldehyde in saturated aqueous mercuric chloride for the preparation of routine paraffin sections which were stained with hematoxylin and eosin.

Blocks were fixed in Carnoy's fluid and 4% basic lead acetate and paraffin sections were prepared for the study of metachromasia. Sections of this material and of formol-sublimate fixed tissues were stained with 0.1% toluidine blue by the method of Bensley(9), dehydrated in alcohol and mounted in Permount. The distribution of the metachromatic substances was identical after all 3 fixatives, although diffuse granularity was present in all the lead acetate-fixed tissues and the Carnoy material cut poorly. For this reason, formol-sublimate fixed tissues were used for subsequent studies.

The solubility in alkali of the various metachromatic substances was tested by incubating deparaffinized sections for varying periods of time in N sodium hydroxide at room temperature. Attempts at extractions of unfixed material were vitiated by the resultant widespread diffusion of metachromatic material throughout the tissues.

The presence of ribonucleic acid and its effect on the staining reaction with toluidine blue was investigated by incubation of deparaffinized sections in 1% ribonuclease (Worthington Biochemicals) for one and two hours(10). The activity of the ribonuclease was confirmed by the ability of the solution to remove the cytoplasmic basophilia of plasma cells.

Sections were also stained by the periodic acid-Schiff method (PAS) of McManus(11) and by Mayer's mucicarmine and mucihematin methods(12). When unexpected PAS negative results were obtained with metachromatic substances, we considered the possibility that treatment of the sections in the process of staining may have removed the reactive substance. Accordingly, some of the sections were counterstained with toluidine blue. Deparaffinized sections were incubated for one hour in 0.1% malt diastase in phosphate buffer at pH 6.0(13) and stained by the PAS method. Control sections of clam were incubated in the buffer only and similarly stained. The efficacy of the enzyme was shown by the ability of the solution to remove glycogen from sections of chicken liver. Deparaffinized sections were incubated at room temperature for 24 hours in an aqueous solution containing 33 mg % of mammalian testicular hyaluronidase (Evans) and stained with toluidine blue. Paraffin sections were prepared of material fixed in cold acetone and stained as follows: 1) by Gomori's revised method for alkaline phosphatase using Fast Blue R.R. and incubating for a period of 2 hours(14), 2) by the

---

* In the paper by Frommhagen and co-workers (8), acknowledgement should have been made of the contributions of Thomas. The authors of paper(8) wish to express their regret at omitting not only the reference by Thomas(7) but also omitting mention of the fact that the idea of using the clam as a source of heparin-like anticoagulants was derived from a personal communication by Dr. Thomas.

Von Kossa method for phosphate-carbonate (15), 3) by Dahl's alizarin red S.(16), and 4) Schujeninoff's method(15) for calcium. Control sections of bone (for calcium) and of kidney (for phosphatase) were run simultaneously. Frozen sections of material fixed in formol sublimate were stained with Baker's sudan Black B(17). A saturated solution (100 mg/cc) of mactin-A was prepared in horse serum, smeared on slides covered with a thin film of gelatin, allowed to dry, and fixed over formaldehyde fumes. Control slides of serum alone were similarly prepared. The smears were then stained with mucicarmine and mucihematin. The effect of hyaluronidase on the anticoagulant was tested by comparing the colors of two aqueous solutions containing 0.4 cc of testicular hyaluronidase (33 mg %) and 0.5 cc of the anticoagulant (800 mg %), to each of which 0.1 cc of a 0.1% aqueous solution of toluidine blue was added before and after incubation for 30 minutes at room temperature.

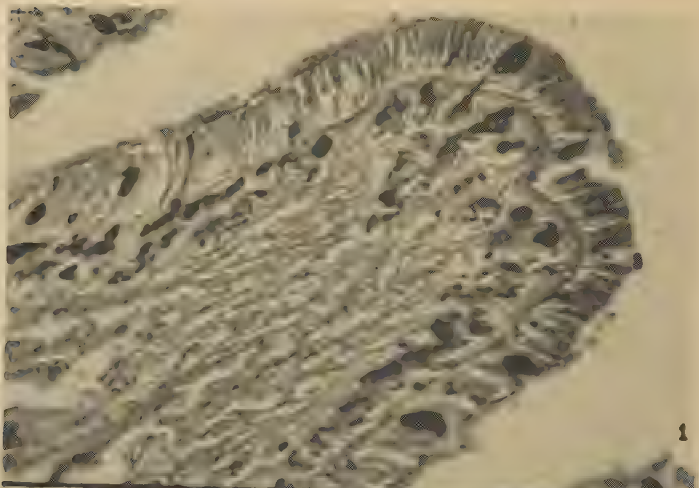
Chemical methods. The periodic acid Schiff test was performed on an aqueous solution of the anticoagulant (mactin-A). The phosphatase activity of water extracts of the mantle tissue of *Mactra solidissima* was determined by the method of Bodansky(18). Inorganic phosphate was determined by the method of Fiske and SubbaRow(19). Clear water-white extracts of the mantle tissues at pH 5 were precipitated by elevation of the pH to 8.5 and the resultant solids analyzed for calcium and phosphate by microchemical methods.

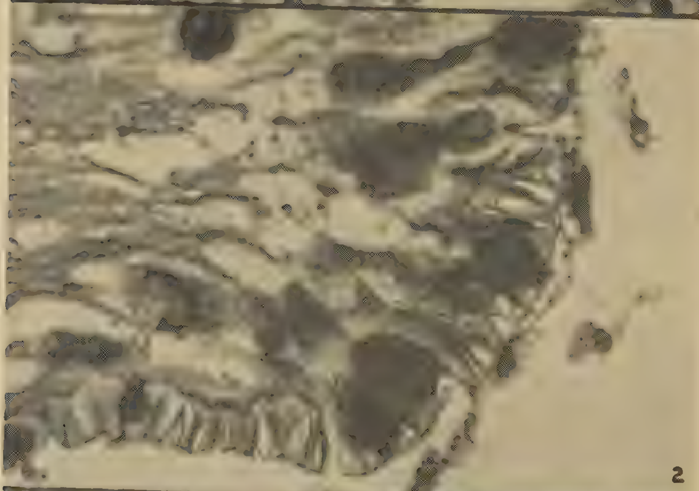
Results. Mast cells are not found in the clam. Four different substances showing intense purple metachromasia are present in abundance: their histochemical properties and those of the anticoagulant (mactin-A) extracted from this species of clam are shown in Table I. Substance A is found in the mucous glands on the inner aspect of the mantle (Fig. 1), on the gills, palps, surface of the body, and in the genital tract. Substance B is observed only in the mucous glands on the outer (shell) side of the mantle (Fig. 2), and the third type (substance C) is found in the mucous glands of the gastrointestinal and urinary tracts. Substance D composes the reticulum fibers and large masses of ground substance in the mantle

TABLE I. Histochemical Properties of the Mucopolysaccharides of the Clam.

	Distribution	Toluidine blue	Ribonuclease + toluidine blue	Hyaluronidase + toluidine blue	Mucihematin	PAS	PAS + diastase	Solubility in N NaOH 2½ hr
Substance A (mantle & body)	Mucous glands of inner mantle, body surface, gills, palps, genital tract	Red-purple	Red-purple	Red-purple	Purple	—*	—	IS§
Substance B (mantle only)	Mucous glands of outer mantle	Blue-purple	”	”	”			S
Substance C (body only)	Mucous glands of gastro-intest., urinary tracts	Red-purple	”	”	Pale purple blue	+	+	IS
Substance D (mantle & body)	Ground substance and reticulin fibers of all tissues	”	”	Pale blue	Pale purple blue	+	+	S
Anticoagulant mactin-A		”	NT‡	” †	Purple	+ †	NT	S

* Occasional trace of pink only. † Test tube reaction. ‡ NT = not tested. § IS = insoluble; S = soluble.
All substances gave positive mucicarmine tests.





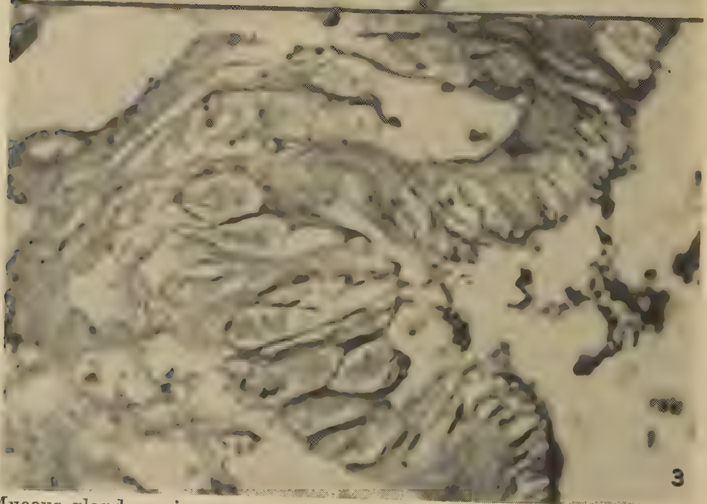


FIG. 1. Mucous glands on inner aspect of mantle. Toluidine blue × 235.
FIG. 2. Mucous glands on shell side of mantle. Toluidine blue × 350.
FIG. 3. Mucous glands on shell side of mantle after alkali extraction. Toluidine blue × 350.

and to a lesser extent in the other tissues of the clam. Extraction with alkali results in the disappearance of metachromatic substances B and D after 2½ hours (Fig. 3) and of all metachromatic substances after more prolonged treatment.

In the test tube the anticoagulant produces intense purple metachromasia which can be greatly reduced by hyaluronidase digestion. The serum controls for the slide tests stain faintly red with mucicarmine and blue with mucihematin; the experimental slides are, however, significantly different, indicating that the anticoagulant is responsible for a deep red color with mucicarmine and an intense purple with mucihematin.

It is not possible to demonstrate any calcium in the clam by Schujeninoff's method. The distribution of calcium by Dahl's alizarin method is identical with that of phosphate-carbonate by the Von Kossa method (Fig. 4 and 5). Considerable amounts of calcium phosphate-carbonate are present beneath the basement membrane of the epithelium on both sides of the peripheral rim of the mantle and, to a much lesser extent, in the form of fine granules in the epithelium and underlying connective tissue (Fig. 5). The largest aggregations are present at the tip of the papillary elevations of the underlying connective tissue (Fig. 5). No calcium is present in the region of the tentacle-like folds at the extreme periphery of the mantle (Fig. 6), which according to Vevelander and Benzer(20) is responsible for the elaboration of the organic matrix of the shell. Chemical analyses of extracts of the mantle indicate the following composition: Calcium 14.57%, phosphate 9.1%. Although carbonate was not determined, it is obvious that the phosphate, if it does exist as the inorganic calcium salt, must predominate.

Phosphatase is present in the same region of the mantle as calcium salts except that it is limited entirely to a small zone at the free border of the epithelial cell (Fig. 7). The presence of phosphatase was verified by the chemical method.

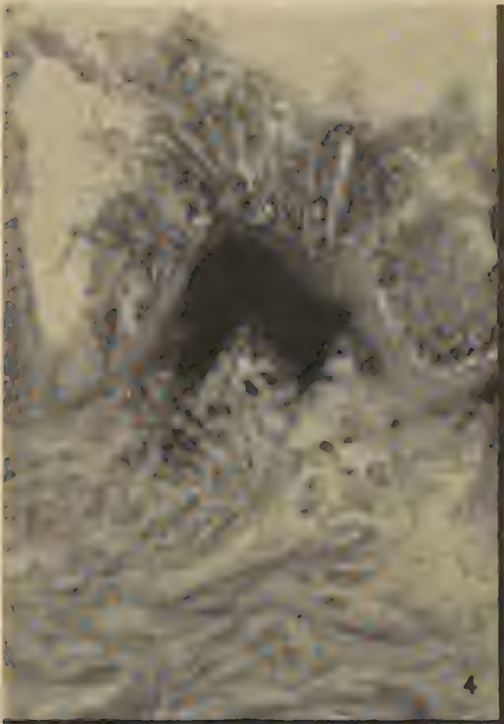
The persistence of metachromatic substances A and B in the tissues after the periodic acid-Schiff test was confirmed by counterstaining with toluidine blue. No sudanophilic lipid or glycogen can be demonstrated in the areas of metachromasia.

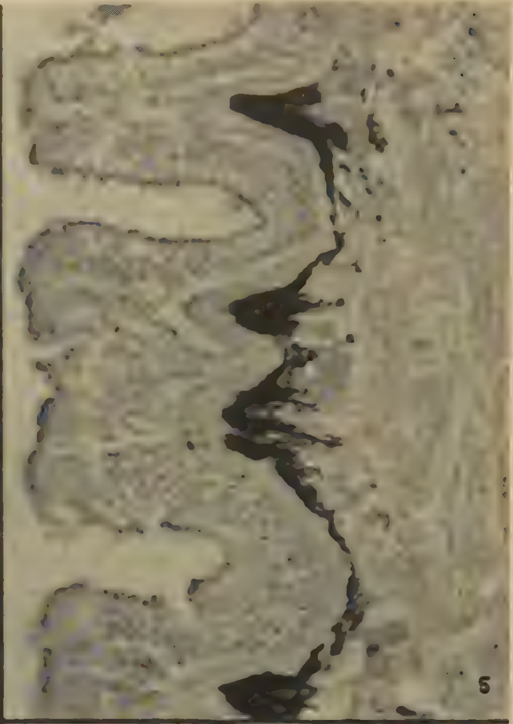
Discussion. Lison(21,22) has demonstrated that alcohol-resistant metachromasia can be due to the presence of sulfuric esters of large molecular size. In the absence of glycogen, ribonucleic acid is the only other substance likely to produce alcohol-resistant metachromasia in paraffin sections. The metachromasia of all 4 substances in the clam is not reduced by ribonuclease; it is therefore due to the presence of acid mucopolysaccharides. The 4 types of acid mucopolysaccharide differ from one another in regard to their solubility in alkali and in hyaluronidase, the availability of the 1-2 glycol groups for oxidation by the PAS method and in the color of their staining reaction with toluidine blue and mucihematin.

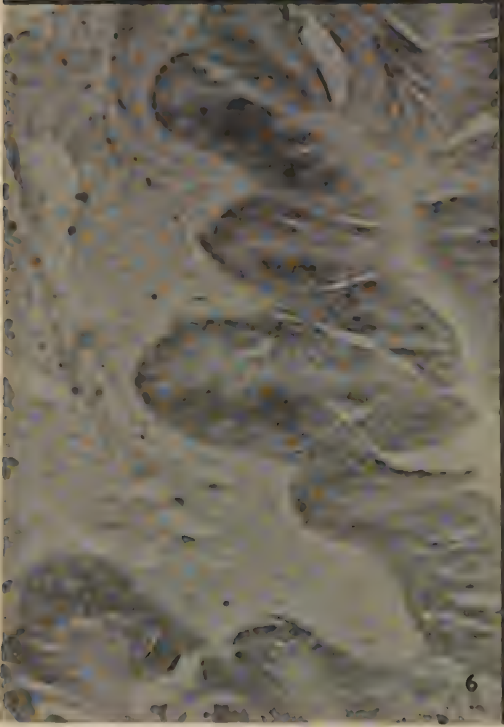
All of the mucopolysaccharides can be removed from the clam by intensive treatment with alkali, so that differential solubility is not of much assistance in determining the source of the anticoagulant (mactin-A). Since the latter is extractable from the body or from the mantle, substances B and C cannot be essential constituents. The anticoagulant is PAS positive and must, therefore, contain substance D (Table I). The presence of substance D in considerable amounts is further confirmed by the ability of hyaluronidase to remove the greater part of the metachromasia from solutions of the anticoagulant.

The very faint reaction of substances A and B with the PAS technic may indicate that they are highly sulfated (or phosphorylated) to such a degree indeed, that very few reactive hydroxyl groups remain(23). Confirmatory evidence for this explanation must await further chemical studies. The PAS positive and PAS negative mucopolysaccharides do not differ significantly in their degree of basophilia or metachromasia. The greater basophilia of the mucin on the shell side of the mantle (Table I) is abolished by treatment with ribonuclease, indicating that in this case at least, ribonucleic acid can even decrease, rather than produce, metachromasia.

Although the mucihematin method of staining is purely empirical, it is of interest to note that the most intense purple color is present in the mucin which is PAS negative. This







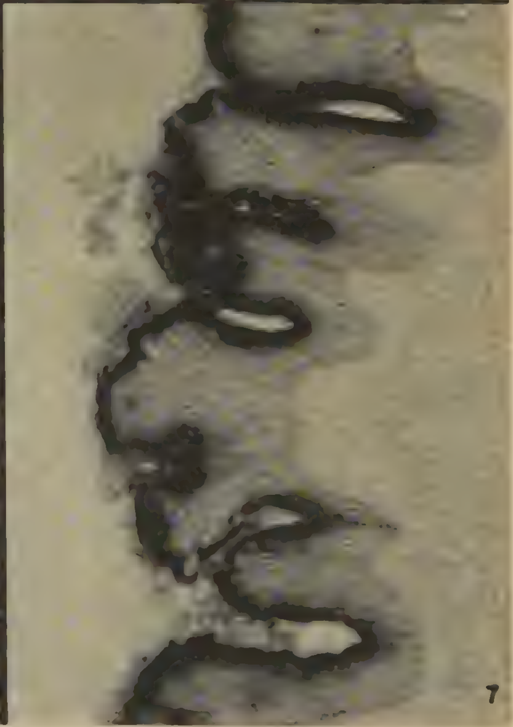


FIG. 4. Calcium deposits, mainly beneath basement membrane of inner aspect of mantle. Dahl's alizarin method × 350.

FIG. 5. Phosphate-carbonate deposits in inner aspect of mantle. Main deposits beneath basement membrane and fine granules in epithelium and subjacent connective tissue. Note

absence of calcium in mucous glands. Von Kossa and neutral red × 350.

FIG. 6. Same section as Fig. 5 showing absence of phosphate carbonate in tentacle-like folds at extreme periphery of mantle. Note absence of mucous glands. Von Kossa and neutral red × 350.

FIG. 7. Alkaline phosphatase in epithelium of inner aspect of mantle. × 350.

would suggest that the purple color with mucihematin is indicative of highly sulfated mucopolysaccharides.

The histological demonstration of calcium phosphate-carbonate was confirmed by chemical procedures which suggested that the phosphate is predominant. Inasmuch as calcium and phosphate were shown to be localized in the same area, it seems probable that the deposits consist of some form of calcium phosphate. The presence of considerable amounts of calcium salts beneath the basement membrane suggests that this may be a storage area for calcium. Contrary to the reports of Bevelander and Benzer(20), it was not possible to demonstrate calcium salts in the mucous glands of the mantle (Fig. 5); it seems probable however, as they suggest, that the mucoid secretion of the glands plays a physical role in the transfer of calcium salts from the mantle to the shell. The calcium of the shell appears to consist mainly of calcium carbonate, whereas that of the mantle is mainly in the form of calcium phosphate(20,24,25). In view of the topographical relationship of phosphatase to the underlying calcium deposits the enzyme may take part in the "secretion" of calcium salts. The affinity of acid mucopolysaccharide for calcium ions and their importance in calcification has been the subject of recent research(26,27). The deeper deposits of inorganic calcium salts in the mantle are present in areas rich in acid mucopolysaccharide, but the overlying epithelium does not contain any of this material. If calcium salts are first formed in the deeper mucoid connective tissue and subsequently "secreted" by the epithelium, they may, as suggested by Thomas(28), be derived from a calcium mucopolysaccharide complex. The role of alkaline phosphatase in this process, is however obscure since mucopolysaccharide cannot be demonstrated in the cells which contain the enzyme, nor is there any evidence of enzymatic activity in the main deposits of calcium salts.

Summary. 1. Heparin-like substances in the clam do not arise from mast cells such as exist in higher vertebrates. 2. Four different acid mucopolysaccharides in the clam are described. The heparin-like anticoagulant (mactin-A) is probably a mixture of all 4 types but appears to consist mostly of the mucopolysaccharides of the ground substance of the connective tissues. 3. Ribonucleic acid is present in association with acid mucopolysaccharide in the mucous glands on the shell side of the mantle. 4. Calcium, phosphate and alkaline phosphatase are present in different sites in the epithelium and subjacent tissues of both sides of the mantle and are not topographically related to the mucous glands.

The authors wish to thank Miss Eugenia Berry for her valuable assistance, and Mr. Leslie McWilliam for the photomicrographs.

---

1. Hammarsten, O., *Arch. ges. Physiol.*, 1885, v36, 412.

2. Levene, P. A., *J. Biol. Chem.*, 1925, v65, 683.

3. Soda, T., and Egami, H., *Bull. Chem. Soc. Japan*, 1948, v13, 652.

4. Soda, T., *Chemical Researches (Japan), Biochem.*, 1948, v1, 51.

5. Masamune, H. T., and Yasouka, T., *Tohoku J. Exp. Med.*, 1947, v49, 177.

6. Immers, J., and Vasseur, E., *Experientia*, 1949, v5, 124.

7. Thomas, L. J., *Biol. Bull.*, 1951, v101, 230.

8. Frommhagen, L. H., Fahrenbach, M. J., Brockman, J. A., Jr., and Stokstad, E. L. R., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 280.

9. Bensley, S. H., *Anat. Rec.*, 1934, v60, 93.

10. Opie, E. L., and Lavin, G. I., *J. Exp. Med.*, 1946, v84, 107.

11. McManus, J. F. A., *Stam. Technol.*, 1948, v23, 99.

12. *McClung's Handbook of Microscopical Technique*, Third Edition, New York, 1950, p. 264.

13. Mowry, R. W., and Borgle, R., *Am. J. Path*, 1950, v27, 611.

14. Gomori, G., *J. Lab. and Clin. Med.*, 1951, v37, 526.

15. ———, *Microscopic Histochemistry*, 1952, p. 32.

16. Dahl, L. K., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 474.

17. Baker, J. R., *Quart. J. Micros. Sci.*, 1944, v85, 1.

18. Bodansky, A., *J. Biol. Chem.*, 1933, v99, 197.

19. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, v66, 375.

20. Bevelander, G., and Benzer, P., *Biol. Bull.*, 1948, v94, 183.

21. Lison, L., *Arch. de Biol.*, 1935, v46, 599.

22. ———, *C. R. Soc. de biol.*, 1935, v118, 821.

23. Jorpes, J. E., Wernor, B., and Aberg, B., *J. Biol. Chem.*, 1948, v176, 277.

24. Biederman, W., *Winterstein's Hndb. d. Verg. Physiol.*, 1914, v3, 319.

25. Plate, L., *Allgemeine Zool. v. Abstammunglehre*, Teile 1, Fisher Jana.

26. Rubin, P. S., and Howard, J. E., *Metabolic Interrelations*, Trans. 2nd Conf., New York, 1950, p. 155.

27. Boyd, E. S., and Neuman, W. F., *J. Biol. Chem.*, 1951, v193, 243.

28. Thomas, L. J., Ph.D., Thesis, Univ. Pennsylvania, 1953.

Received July 7, 1953. P.S.E.B.M., 1953, v83.

Influence of Cortisone on Leptomeningeal Reaction Induced by Talc.* (20511)

JACK H. OPPENHEIMER† AND WALTER H. RIESTER.‡ (Introduced by C. Ragan.)

From the Departments of Medicine and Surgical Pathology, Columbia University College of Physicians and Surgeons, New York.

Ragan and co-workers(1) have demonstrated a marked retardation in the wound healing of rabbits treated with large doses of cortisone. This observation has been extended to the inflammatory and reparative processes of other mesodermal structures. Blunt(2) has studied delayed healing in experimental fractures of rabbits. Ducommun(3) and Scheinberg and Saltzstein(4) showed that ACTH and cortisone prevented the formation of intra-abdominal adhesions following the intraperitoneal instillation of talc, while Scheinberg, Saltzstein and Johnson(5) observed similar results in the prevention of pleural and pericardial adhesions.

Because cortisone diminished the inflammatory response of other serous surfaces, a study of its action on the meningeal reaction to the intrathecal administration of a sterile irritant was undertaken. Of several substances tried talc was the most effective in eliciting a meningeal reaction.

It was also felt that control of the meningeal reaction might be of value in the treatment of tuberculous meningitis as well as other granulomatous affections of the meninges. In such instances it seemed possible that the reduction of exudation and inflammatory response would make the offending organism more accessible to the action of the chemotherapeutic agent. Furthermore, the use of cortisone in the prevention of post-traumatic adhesions also suggested itself.

Materials and method. Eighteen white male rabbits weighing from 3.0 to 4.8 kg received a cisternal injection of 1 cc of a saline suspension of talc after a similar quantity of cerebrospinal fluid had been withdrawn. The talc suspension was sterilized by autoclaving. Ten animals were treated with cortisone, while 8 served as controls. In the cortisone-treated group 4 animals received a cisternal injection of 1 cc of a 1:9 suspension of talc, while 6

---

* Supported in part by the Masonic Foundation for Medical Research and Human Welfare and the U. S. Public Health Service (grant A-21).

† Now at the Boston City Hospital, Div. V & VI (Boston University), Boston, Mass.

‡ Now at the Philadelphia General Hospital, Philadelphia, Pa.

The authors wish to acknowledge the invaluable and generous assistance of Drs. Charles Ragan, Department of Medicine, and Raffael Lattes, Department of Surgical Pathology, Columbia University College of Physicians and Surgeons.

Hydrocortisone acetate was supplied through the courtesy of Dr. Elmer Alpert of Merck and Company, Rahway, N. J.

received a 1:4 suspension. In the control series 2 animals were injected with a 1:9 suspension, whereas a 1:4 suspension was employed in the remaining 6.

The 10 treated rabbits received daily intramuscular injections of a saline suspension of cortisone acetate (Merck) for 15 days after the intrathecal administration of talc. Eight of these rabbits received cortisone for 2 days prior to the cisternal injection, while 2 received no such pretreatment. Nine rabbits received 10 mg cortisone per kilogram and one 5 mg/kg intramuscularly.

All cisternal injections were carried out under nembutal anesthesia. The talc suspension was administered slowly (15-20 seconds) to avoid respiratory arrest resulting from acute pulmonary edema(6).

All animals were sacrificed 15 days after injection. The brain and meninges were fixed in 10% formalin. Sections were stained with hematoxylin and eosin.

Results. Following cisternal injection, transient tonic and clonic convulsions were observed in several rabbits shortly after the talc administration. No permanent neurological lesions were seen. In no instance was evidence of meningeal infection found.

The average weight loss of the cortisone-treated rabbits was 625 g, whereas that of the control rabbits was 314 g. Although the difference in the mean weight loss appears significant, it must be pointed out that certain control rabbits lost more weight than some of the cortisone-treated animals. However, all the cortisone-treated animals appeared markedly lethargic and emaciated after 2 weeks.

In this series of experiments 2 animals died while receiving cortisone. One, treated with 10 mg/kg, died on the 7th day after intrathecal injection, and the other, on 5 mg/kg, died on the 11th day. The exact cause of death could not be determined in either instance.

In the control group there was diffuse thickening and opacity of the meninges, especially in the area of the brain stem, whereas in the treated animals the translucency of the normal meninges was better preserved. The differences observed grossly were not as striking as those seen histologically.

Microscopic sections from the control group revealed primarily a phagocytic, histiocytic response with some fibroblastic and minimal angioblastic proliferation. Foreign-body giant cells appeared to engulf individual talc crystals, and dense adhesions were present in certain locations between pia and arachnoid membranes. The talc particles were easily visualized under polarized light because of the doubly refractile quality of the crystals (Fig. 1).

In contrast to the controls, the meningeal response of the treated animals was consid-

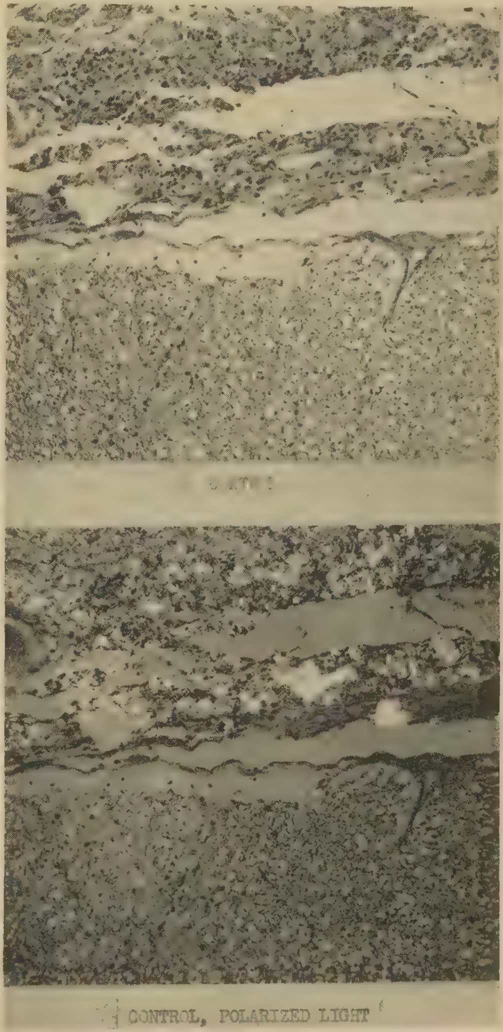


FIG. 1. Photomicrograph of leptomeningeal reaction in an untreated rabbit 15 days after cisternal injection of sterile talc. Note fibroblastic and phagocytic response. When viewed under polarized light, talc crystals may be seen. × 105.

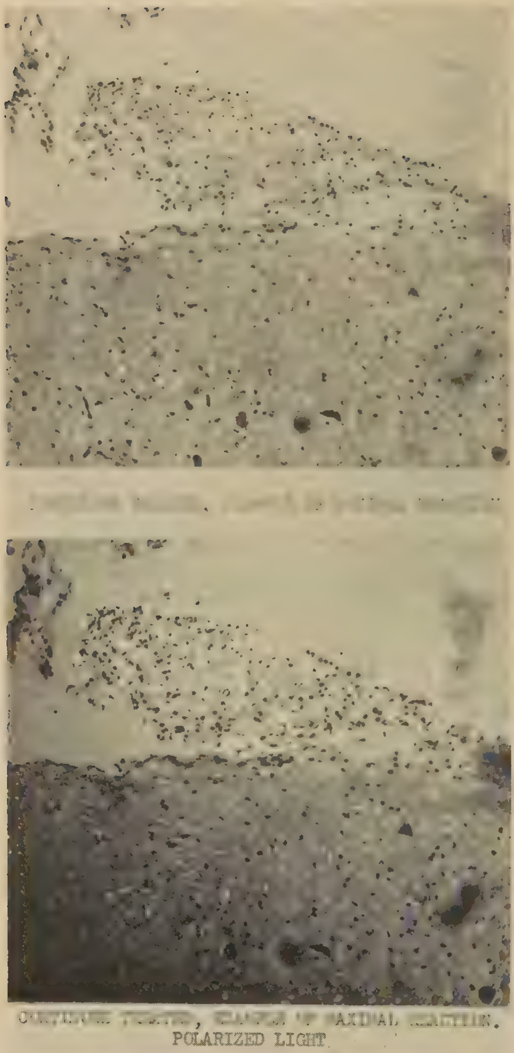


FIG. 2. Maximal reaction to talc seen in a rabbit treated 2 days before and 15 days after cisternal injection of talc with 10 mg cortisone/kg. There is minimal meningeal reaction. Talc crystals may be seen under polarized light. × 105.

erably diminished, although the degree of reaction varied from animal to animal. The extent of this variation is indicated in Fig. 2 by examples of maximal and, in Fig. 3, by the minimal meningeal response in cortisone-treated animals. Often loose collections of talc crystals could be seen lying free in the subarachnoid space without evident reaction (Fig. 3). Even when the presence of talc elicited a histiocytic response, individual crystals were generally not engulfed by phagocytes. No adhesions between pia and arachnoid were established.

Evaluation of the degree of meningeal reactivity was entirely subjective, and no attempt to quantitate the cellular response was made.

Preliminary experiments with intrathecal hydrocortisone. To avoid the systemic toxicity of parenterally administered cortisone, the use of intrathecal hydrocortisone suggested itself. Accordingly, using sterile precautions,

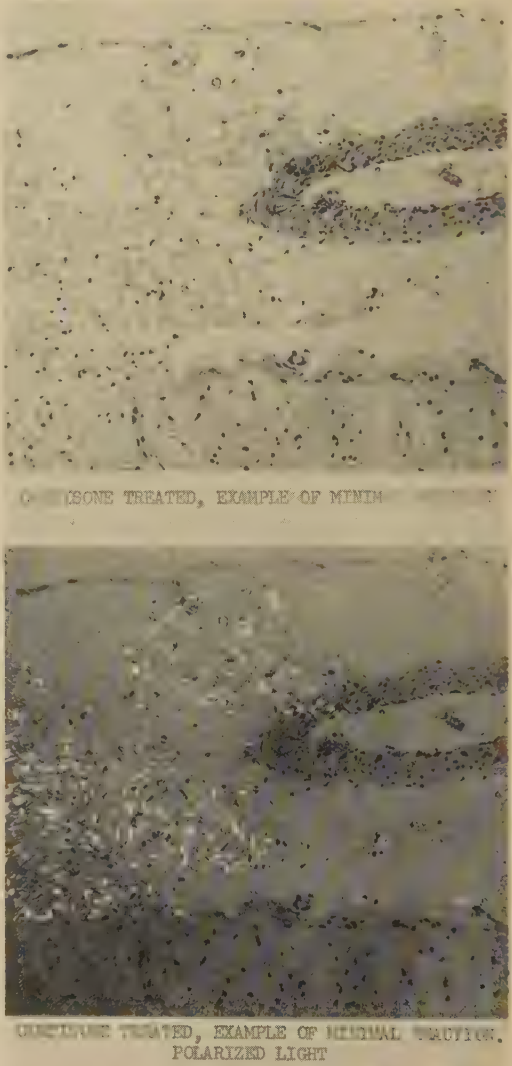

CORTISONE TREATED, EXAMPLE OF MINIMAL REACTION
CORTISONE TREATED, EXAMPLE OF MINIMAL REACTION.
POLARIZED LIGHT

FIG. 3. Minimal reaction to talc seen in a rabbit treated similarly to that shown in Fig. 2. There is almost complete absence of meningeal reaction. Talc crystals may be seen under polarized light. × 105.

3 animals received 10 mg (0.4 cc) of a saline suspension of hydrocortisone acetate (Merck) as well as 1.0 cc of a 1:4 sterile saline suspension of talc in a single intrathecal injection. On the 7th day another dose of 10 mg hydrocortisone was administered by the same route. In order to assess the effect of the hydrocortisone suspension on the meninges, 3 animals received 10 mg of hydrocortisone plus 1.0 cc saline, followed on the 7th day by another equal dose of hydrocortisone. Following the intrathecal administration of hydrocortisone both with and without talc, the animals showed striking convulsive behavior, extensor rigidity, and marked tachypnea for approximately 1 hour following the injection. There was complete recovery in every animal, and no residual neurological abnormalities were noted. No attempt was made to determine whether the hydrocortisone or the vehicle, including 0.9% benzyl alcohol, was responsible for the immediate clinical reaction. One of the animals injected with talc and hydrocortisone died on the 12th day; the cause of death was not ascertained.

The microscopic appearance of the meninges of the 2 surviving animals injected with talc and hydrocortisone showed some diminution of the meningeal reaction, although this effect was not as striking as with cortisone. The animals, however, did not show the emaciation and lethargy characterizing those treated with cortisone, and the weight loss averaged 240 g as compared to a mean weight loss of 625 g in the cortisone group. The gross and microscopic appeance of the meninges of those animals injected only with hydrocortisone was not abnormal.

Summary. Eighteen rabbits received intrathecal injections of 1.0 cc of talc suspension and were sacrificed 15 days later. Eight rabbits were untreated, serving as controls. These developed a meningeal reaction consisting of the appearance of histiocytes, macrophages, and giant cells. Ten animals treated with daily injections of 5 and 10 mg cortisone/kg showed a marked diminution of the meningeal response. Preliminary experiments with the use of hydrocortisone, injected intrathecally (10 mg) on the 1st and 7th days, also suggest diminution of the meningeal response to talc, though not as striking as with the use of intramuscular cortisone.

---

1. Howes, E. L., Plotz, C. M., Blunt, J. W., and Ragan, C., *Surgery*, 1950, v28, 177.

2. Blunt, J. W., Jr., Plotz, C. M., Lattes, R., Howes, E. L., Meyer, K., and Ragan, C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 678.

3. Ducommun, P., and Mach, R. S., *Semaine des Hopitaux*, 1951, v26, 3170.

4. Scheinberg, S. R., and Saltzstein, H. C., *Arch. Surg.*, 1951, v63, 413.

5. Scheinberg, S. R., Saltzstein, H. C., and Johnson, A. S., *Post-graduate medicine and surgery:* Surgical forum, Am. Coll. Surg., Philadelphia, Saunders, 1951, pp. 531-536.

6. Cameron, G. R., and De, S. N., *J. Path. and Bact.*, 1949, v61, 375.

Received July 16, 1953. P.S.E.B.M., 1953, v83.

Effects of Pressor and Depressor Agents on Pulmonary and Systemic Pressures of Normotensives and Hypertensives.* (20512)

SOL FORMAN, L. G. MAY, ALENE BENNETT, MITSU KOBAYASHI, AND RAYMOND GREGORY.

From the John Sealy Memorial Laboratory for Clinical Research and the Department of Internal Medicine, University of Texas School of Medicine, Galveston.

Hypertrophy of the right ventricle does not occur in uncomplicated essential hypertension (1). This well established observation indicates that pulmonary artery pressure is normal in hypertensive patients. Cournand(2) found normal right ventricular pressures in 5 hypertensives and used this as evidence that the pulmonary arterial system lacked vasomotor function comparable to that of the systemic arterioles. Normal pulmonary arterial pressures have been observed in a few hypertensives by others(3-6).

TABLE I. Effect of Epinephrine, Angiotonin, Nor-epinephrine, and Tetraethylammonium Bromide on Pulmonary and Brachial Pressures of Normotensive Patients.

Patient	Age	Sex	Pulmonary*		Brachial*		Pulse	Pulmonary		Brachial		Pulse	Pulmonary		Brachial		Pulse
			S/D	Mean	S/D	Mean		S/D	Mean	S/D	Mean		S/D	Mean	S/D	Mean	
			Control					Epinephrine					Angiotonin				
WJ	42	♂	29/16	22	100/70	85	100	40/22	30	130/ 70	100	120	12/7	9	140/100	120	60
JB	22	♂	12/0	6	120/60	90	94	30/0	15	160/ 80	120	140	24/9	14	158/100	129	80
PA	24	♂	41/25	27	100/60	80	60	45/30	40	180/110	145	120					
EP	47	♀	20/10	15	110/65	87	60	40/15	20	140/ 90	125	80	30/15	23	160/100	135	60
MH	40	♀	43/6	20	120/70	95	80	77/25	50	140/ 80	110	100	67/18	34	140/110	125	80
JT	31	♂	13/4	8	120/80	100	100	40/12	18	190/ 90	140	80	38/12	22	190/120	155	60
LD	40	♂	16/5	10	100/60	80	80	20/7	14	120/ 50	85	100	25/10	16	160/90	125	60
CM	51	♂	25/7	13	94/60	77	80	35/8	20	160/ 80	120	120	43/18	28	160/140	150	80
JR	57	♂	15/5	8	104/58	81	80	18/7	10	140/ 70	105	100	20/5	12	160/90	125	80
EW	40	♀	14/4	8	100/70	80	80	23/5	12	120/ 60	85	100	27/10	18	150/110	130	70
								Nor-epinephrine					Tetraethylammonium bromide				
ES	60	♂	17/7	12	110/60	85	80	34/18	25	200/100	150	60	15/0	9	110/60	85	100
VD	52	♂	20/5	14	90/70	85	80	27/10	18	140/ 90	115	50	3/0	0	74/60	67	120
AC	26	♂	12/1	5	120/80	100	110	30/10	18	200/120	160	60	8/0	3	100/48	90	160
LH	48	♂	5/0	1	80/50	65	70	5/0	3	140/ 78	109	50					
AG	30	♀	10/0	3	120/84	102	120	15/0	6	190/100	145	120	1/0	0	100/48	74	160
HB	66	♂	9/1	4	80/60	70	60	19/6	12	140/ 80	110	80					

Dosage: Epinephrine = 0.025 mg I.V.; angiotonin = 5·7 cc I.V.; nor-epinephrine = 0.025 mg I.V.; tetraethylammonium bromide — 300 mg I.V.
* mm Hg.

TABLE II. Effect of Epinephrine, Angiotonin, Nor-epinephrine, and Tetraethylammonium Bromide on Pulmonary and Brachial Pressures of Hypertensive Patients.

Patient	Age	Sex	Pulmonary*		Brachial*		Pulse	Pulmonary		Brachial		Pulse	Pulmonary		Brachial		Pulse
			S/D	Mean	S/D	Mean		S/D	Mean	S/D	Mean		S/D	Mean	S/D	Mean	
			Control					Epinephrine					Angiotonin				
QM	28	♀	12/2	5	138/100	129	84	30/5	17	180/120	150	120	25/9	15	190/140	165	80
NL	41	♂	12/2	4	194/110	152	80						50/25	38	220/150	185	100
								Nor-epinephrine					Tetraethylammonium bromide				
ER	52	♂	10/0	4	160/100	130	100	42/17	26	280/140	210	50	4/0	1	140/100	120	140
PL	39	♀	18/6	10	190/120	155	100						11/3	6	134/100	117	90
NL	41	♂	12/2	4	194/110	152	80						3/0	0	160/120	140	120
EJ	37	♀	25/12	13	200/130	165	120										
WW	55	♂	15/3	9	180/120	150	60										

Dosage: Epinephrine = 0.025 mg I.V.; angiotonin = 5-7 cc I.V.; nor-epinephrine = 0.025 mg I.V.; tetraethylammonium bromide = 300 mg I.V.
* mm Hg.

It was the purpose of this study to measure the pressure in the pulmonary artery in normotensive and uncomplicated hypertensive patients. If the pulmonary artery pressure was the same in normotensives and hypertensives, it was then proposed to compare the responses of the brachial and pulmonary arterial pressures to pressor and depressor agents in these two groups. It was believed that this would yield information pertinent to an understanding of the pathogenesis of essential hypertension.

Pulmonary and brachial arterial pressures of 16 normotensive and 7 hypertensive subjects were studied. The responses of these pressures to angiotonin,* epinephrine, nor-epinephrine,* and tetraethylammonium bromide* were measured. Brachial pressure was obtained by the auscultatory method. The pulmonary artery was catheterized and the pressure was recorded by a Sanborn Electromanometer and Viso-Cardiette. Our usual sedation for this procedure was employed(7).

Results. The data are presented in Tables I and II. They show that the pulmonary artery presure in all normotensives was within the normal range established by Dexter(4) with the exception of patients P. A. and M. H. The elevated pulmonary artery pressure of P. A. is unexplained. Patient M. H. has chronic pulmonary heart disease secondary to pulmonary emboli. These 2 patients have been left in our series because their brachial artery pressures were normal.

The pulmonary artery pressure rose in normotensives whenever epinephrine, nor-epinephrine, or angiotonin caused an elevation of systemic arterial pressure except in W. J. This subject's pulmonary artery pressure fell from 22 to 9 mm Hg with angiotonin. Tetraethylammonium bromide caused a fall in pulmonary artery pressure in all normotensive individuals(8).

Table II shows that hypertensive subjects have normal pulmonary artery pressures. Angiotonin, epinephrine, and nor-epinephrine always produced an elevation of pulmonary arterial pressure in hypertensives if increased systemic pressures resulted from these agents. Tetraethylammonium bromide produced a fall in both pulmonary and systemic artery pressures in hypertensives.

Discussion. We have found that the pulmonary artery pressure in hypertensives is within normal limits (Table II). The response of systemic and pulmonary artery pressures to epinephrine, nor-epinephrine, angiotonin, and tetraethylammonium bromide is the same in hypertensives and normotensives. From this, we have concluded that the vasomotor mechanisms which control the pulmonary arterial and systemic arterial systems are similar if not identical. Inasmuch as the pulmonary artery pressure is normal in hypertensives and increases with epinephrine, nor-epinephrine, and angiotonin, it is improbable that these or any similar substances are responsible for the systemic blood pressure elevation of essential hypertension.

The effect of angiotonin is noteworthy because this substance has been considered so widely to be the cause of the elevated blood pressure in both experimental renal hypertension and essential hypertension. Inasmuch as pulmonary artery pressure is the same in subjects with essential hypertension and in individuals with normal arterial pressure and since angiotonin raises pulmonary and systemic arterial pressure of hypertensive subjects, it is concluded that angiotonin is not the cause of systemic artery hypertension in patients with essential hypertension.

Current opinions concerning whether the terminal pulmonary vascular branches contain constrictor components are conflicting(4,1,9, 10). The known anastomoses(11) of the pulmonary capillary bed with the bronchial arterial system also introduces factors which make difficult the precise interpretation of mechanisms involved in these effects. For these reasons further studies are in progress to elucidate the responsible mechanisms.

Summary and conclusions. The pulmonary and brachial artery pressures and their responses to pressor and depressor agents have been studied in normotensive and hypertensive subjects. Pulmonary artery pressure is within the same range in both groups. The response of pulmonary artery pressure to pressor and depressor agents used is also the same in both groups. It is emphasized that the elevation of pulmonary artery pressure due to angiotonin, epinephrine, and nor-epinephrine is the same in hypertensive and normotensive subjects. It is concluded that none of these or any similarly acting substances plays a part in sustaining the blood pressure elevation in patients with chronic essential hypertension.

---

* Dr. Oscar H. Helmer of the Lilly Laboratory for Clinical Research supplied the *angiotonin* and *tetraethylammonium bromide* used in these experiments, and Dr. Kenneth M. Smoot of the Department of Medical Research, Winthrop Stearns Inc. supplied the *Levophed®*. We are grateful for the generous amounts of both these substances.

1. Brenner, O., *Arch. Int. Med.*, 1935, v56, 211, 457, 724, 976, 1189.

2. Bloomfield, R. A., Lauson, H. D., Cournand, A., Breed, E. S., and Richards, O. W., *J. Clin. Invest.*, 1946, v25, 639.

3. Freis, E. D., Stanton, J. R., Culbertson, J. W., Litter, J., Halperin, M. H., Burnett, C. H., and Wilkins, R. W., *J. Clin. Invest.*, 1949, v28, 353.

4. Dexter, L., Dow, J. W., Haynes, F. W., Whittenburger, J. L., Ferris, B. G., Goodale, W. T., and Hellems, H. K., *J. Clin. Invest.*, 1950, v29, 602.

5. Freis, E. D., Stanton, J. R., Litter, J., Culbertson, J. W., Halperin, M. H., Moister, F. C., Wilkins, R. W., *J. Clin. Invest.*, 1949, v28, 1387.

6. Werko, Lars and Logerlof, Henrik, *Acta Med. Scand.*, 1949, v133, 427.

7. May, L. G., Bennett, Alene, Lane, A. L., Futch, E. D., Schoomer, Mary Lynn, and Gregory, Raymond, *Proc. Cent. Soc. Clin. Research*, 1948, v21, 44.

8. Green, D. G., and Bunnell, I. L., *J. Clin. Invest.*, 1950, v29, 818.

9. Hellems, H. K., Haynes, F. W., and Dexter, L., *J. Applied Physiol.*, 1949, v2, 24.

10. Fowler, N. O., Westcott, R. N., Hauenstein, V. D., Scott, R. R., and McGuire, J., *J. Clin. Invest.*, 1950, v29, 1387.

11. Miller, W. S., *The Lung*, Charles C. Thomas, Springfield, Illinois, 2nd Edition, 1950.

Received July 27, 1953. P.S.E.B.M., 1953, v83.

Prevention of Dehydroascorbic Acid Diabetes by Atropine.* (20513)

JOHN W. PATTERSON.

From the Department of Anatomy, School of Medicine, Western Reserve University, Cleveland, O.

Dehydroascorbic acid, the reversibly oxidized form of ascorbic acid, produces permanent diabetes in rats(1) and rabbits(2) when multiple large doses are injected intravenously. Immediately following a single intravenous injection of dehydroascorbic acid, there is marked stimulation of the autonomic nervous system which is characterized by salivation, lacrimation, and a rise in blood pressure(3). Atropine administered before the injection of dehydroascorbic acid prevents these effects. Atropine also prevents the diabetogenic effect of dehydroascorbic acid. This paper is concerned with a consideration of this phenomenon.

Experimental. Male Sprague-Dawley rats weighing 108-175 g were injected intravenously with 0.2 g per kilo of dehydroascorbic acid followed in one hour by 0.7 g per kilo on each of 3 consecutive days. The rats receiving atropine sulfate were injected intravenously with 50 mg per kilo of the drug 20 minutes before each of the 0.7 g per kilo injections of dehydroascorbic acid. Those rats with blood sugars greater than 200 mg per 100 cc of blood were considered diabetic. At the end of 2 weeks 8 out of 13 control rats had diabetes, whereas only 1 out of 12 rats receiving atropine developed diabetes (Table I). The P value for this difference is 0.001 indicating that the effect of atropine is highly significant.

A similar experiment was performed on rats that had undergone splenectomy 8-10 days before the injection of dehydroascorbic acid and atropine. In this series (Table I) the atropine did not prevent diabetes.

Discussion. The fact that splenectomy reverses the effect of atropine in preventing dehydroascorbic acid diabetes suggests a peripheral locus for this action of atropine. It may be mediated by a substance produced in

---

* Aided in part by the Cleveland Diabetic Fund.

TABLE I. Effect of Atropine and Splenectomy on the Development of Dehydroascorbic Acid Diabetes.

	Intact rats		Rats with splenectomy	
	Atropine	Control	Atropine	Control
No.	12	13	5	4
Wt (g)				
Range	108–165	120–175	127–162	135–170
Avg	126	150	147	153
% diabetic				
2 days	58	100	100	100
1 wk	8	84	80	75
2 "	8	61	80	75
Avg 2 wk blood sugar (mg/100 cc)				
Diabetic	260	430	400	390
Non-diabetic	122	140	125	190

the spleen or, since the pancreas and spleen are supplied by a common artery, it may be the result of a change in the relative amount of blood and diabetogenic agent flowing through each organ. The latter of these suggestions seems more probable. Changes in the blood supply to the pancreas are known to prevent alloxan diabetes. The injection of adrenalin(4) or the clamping of the renal pedicle(5) are effective. The production of diabetes with dehydroascorbic acid is similar to injecting alloxan and adrenaline simultaneously. Only a small amount of the diabetogenic agent reaches the islet tissue. Atropine is known to reverse the sympathomimetic effect of dehydroascorbic acid(3). If this reversal is greater in the spleen than in the pancreas, blood would be shunted through the spleen with a decrease in the amount of dehydroascorbic acid reaching the islets. It is claimed that the injection of adrenaline following the administration of a blocking agent causes a dilatation of the splenic vessels(6). If the injection of dehydroascorbic acid produces a similar effect in the atropinized rat the shunting of blood past the pancreas would be even greater.

Summary. The administration of atropine before the intravenous injection of dehydroascorbic acid prevents diabetes. This effect of atropine is not observed in splenectomized rats.

---

1. Patterson, J. W., *J. Biol. Chem.*, 1950, v183, 81.

2. Princiotto, J. W., *J. Clin. Endocr.*, 1951, v11, 775.

3. Patterson, J. W., *Am. J. Physiol.*, 1951, v167, 119.

4. Carrasco-Formiguera, R., and Escobar, I., *Am. J. Physiol.*, 1948, v152, 609.

5. Houssay, B. A., and Martinez, C., *Nature*, 1948, v162, 571.

6. Lippay, F., Mitchell, G. G., and Potter, B. J., *Austral. J. Exp. Biol. Med. Sci.*, 1950, v28, 517.

Received July 27, 1953. P.S.E.B.M., 1953, v83.

Inhibition of Mumps Virus Multiplication by a Synthetic Polypeptide.* (20514)

MAURICE GREEN AND MARK A. STAHMANN.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

Synthetic lysine polypeptides have been demonstrated to reduce tobacco mosaic virus infectivity(1), to inhibit the growth of influenza virus in the chick embryo(2), to inhibit the multiplication of a bacteriophage(3), and to protect embryonated eggs infected with several strains of infectious bronchitis virus and Newcastle disease virus(4). In the present communication it will be shown that synthetic lysine polypeptides markedly reduce the susceptibility of the chick embryo to infection with mumps virus. Inhibition of mumps virus multiplication in the allantoic sac was obtained when very small amounts of polypeptides were injected either in the allantoic sac or yolk sac. Striking reduction in virus proliferation was obtained when the polypeptide was administered either 1 hour before infection or as late as 36 hours after infection with mumps virus. Evidence is presented to show that the virus *per se* is not demonstrably inactivated by the lysine polypeptides.

Materials and methods. (a) *Virus.* The Enders strain of mumps virus[†] used in these studies has undergone an unknown number of passages in monkeys' parotid glands, and 12 amnion and 36 allantoic sac passages in embryonated eggs. The virus was cultivated in the allantoic sac of 7-day old embryos which were incubated at 37°C for 6 days after inoculation. The allantoic fluids were harvested after preliminary chilling at 4°C for several hours. Virus inoculum pools were prepared as a 10% solution of infected allantoic fluid in sterile horse serum (previously heated at 56°C for 30 minutes). The preparation was divided into several aliquots which were stored at –70°C. Individual aliquots were then used and discarded.

(b) *Polypeptides.* The lysine polypeptide hydrochlorides[‡](5) were dissolved in physiological saline and these solutions adjusted to pH 6.3 with alkali. The glutamic acid polypeptide(6) was dissolved in an equivalent amount of alkali and the solution adjusted to pH 7.3. All solutions were passed through a Seitz filter and tested for bacterial sterility. The maximum non-lethal dose of the lysine polypeptides was about 1.0 mg per chick embryo and of the glutamic acid polypeptide greater than 20 mg per embryo.

(c) *Virus infectivity titrations.* Serial 10-fold dilutions of infected allantoic fluid were made in 0.85% saline and 0.1 cc amounts injected into groups of 4 eggs intra-allantoically. After 6 days incubation at 37°C, each allantoic fluid was removed after preliminary chilling and tested for its ability to agglutinate chicken erythrocytes. Virus infectivity titration end points (I.D. 50) were calculated by the 50% end point method of Reed and Muench(7).

(d) *Virus hemagglutination titrations.* Serial 2-fold dilutions of infected allantoic fluid were prepared in 0.85% saline, and to 0.5 cc of each dilution in 10 x 75 mm pyrex culture tubes was added 0.25 cc of a 1% suspension of washed chicken erythrocytes. The tubes were read after standing 1 hour at room temperature and the end point was taken as the highest dilution at which complete or partial agglutination of the red blood cells occurred.

(e) *Reproducibility of hemagglutination titration end points.* To evaluate accurately the significance of differences in hemagglutination titers between control and treated groups of embryos, it was essential to calculate the reproducibility of titers under uniform conditions. Therefore, the standard error of

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a research grant from the National Institutes of Health, United States Public Health Service.

[†] We would like to thank Dr. N. S. Khoobyarian for a culture of mumps virus.

[‡] We are indebted to Dr. R. R. Becker for the lysine polypeptides.

TABLE I. Reduction of Hemagglutination Titer of Mumps Virus in Allantoic Fluid of Chick Embryos Injected with Polylysine.

		Hemagglutination titer*						
1st inj., .1 cc intra-allantoic	2nd inj., .1 cc intra-allantoic (1 hr later)	Individual allantoic fluids				Geometric mean	Log mean	Difference from control log
	E.I.D.†							
Saline	10	128	256	32	16	65	1.81	
.4 mg PL‡	”	0	0	0		0		1.81
2 mg PGA§	”	256	256	256	16	128	2.11	0
Saline	30	512	256	128	128	224	2.35	
.4 mg PL	”	4	0	0		2	.20	2.15
Saline	300	320	320	160	160	221	2.33	
.4 mg PL	”	0	32	32	2	7	.83	1.50
Saline	3000	128	128	64		103	2.01	
.4 mg PL	”	8	0	8		4	.60	1.41
Saline	30000	256	256	256	256	256	2.41	
.4 mg PL	”	8	16	8	0	6	.75	1.66
Saline	300000	64	256	256	256	170	2.23	
.2 mg PL	”	32	64	16	64	38	1.58	.65‖

* Expressed as the reciprocal.

† E.I.D. = embryo infectious doses.

‡ Polylysine of avg molecular wt 4900.

§ Polyglutamic acid of avg molecular wt 4800.

‖ Statistical analysis by t test shows that 0.65 log unit difference was highly significant and would occur by chance less than 1 out of 50 times.

the geometric means of the hemagglutination titers of 9 groups of 3 or 4 embryos infected with 30 infectious doses of mumps virus was determined. The standard deviation of the distribution of end points (geometric mean of each group of infected embryos) was 0.24 log unit.

Experimental. In all experiments, fertile White Leghorn eggs were employed. The concentration of virus in the allantoic fluids was measured by the hemagglutination technic 6 days after infection with the virus(7). Ginsberg, Goebel, and Horsfall(8) have shown that hemagglutination is caused by the mumps virus particle itself and that the hemagglutination titer is a direct measure of virus concentration.

(a) *Effect of polylysine on chick embryos infected with various levels of mumps virus.* The experiments presented in Table I were carried out to determine the extent to which the administration of polylysine would inhibit the multiplication of mumps virus in the chick embryo. Groups of 3 or 4 embryos were injected with 0.2 or 0.4 mg of polylysine intra-allantoically. Control embryos were injected with saline. Levels of mumps virus ranging from 10 to 300,000 infectious doses were then inoculated 1 hour later by the same route. After 6 days incubation at 37°C, the eggs were harvested and the hemagglutination titers of the individual allantoic fluids determined. It is evident from Table I that the mean hemagglutination titers of the allantoic fluids from each group of embryos which had received polylysine and had been subsequently infected with from 10 to 30,000 infectious doses of mumps were strikingly lower than the controls. At these levels of virus only 0.7-4% of the maximum concentration of virus was produced in the presence of polylysine as measured by hemagglutination. The multiplication of mumps virus was significantly reduced even when 300,000 infectious doses of virus were injected. Polyglutamic acid of the same molecular weight had no effect on mumps virus multiplication.

There was no demonstrable hemagglutination in many of the infected embryos treated with polylysine. It has been previously shown (8) that virus titers of the order of $10^{-4.30}$ or higher are necessary before hemagglutination is demonstrable with mumps virus. Thus the zero values for the hemagglutination titers of many of the polylysine treated embryos could represent virus titers values ranging from 0

TABLE II. Reduction of Both Hemagglutination and Infectivity Titers of Mumps Virus in Allantoic Fluid of Chick Embryos Injected with Polylysine.

1st inj., .1 cc intra-allantoic	2nd inj., .1 cc intra-allantoic (1 hr later)	Hemagglutination titer of allantoic fluid	Infectivity virus titration end point ID_{50}, log
.2 mg PL*	30 E.I.D.	0	–3.7
"	"	0	–2.7
"	"	0	–4.0
			–3.5 Avg
Saline	30 E.I.D.	1:256	–7.5
"	"	1:256	
"	"	1:128	
			–4.0 Difference

* Polylysine of avg molecular wt 4900.

TABLE III. Effect of Different Quantities of Polylysine on Hemagglutination Titer of Allantoic Fluids Obtained from Chick Embryos Inoculated with 30 E.I.D. of Mumps Virus.

1st inj., .1 cc intra-allantoic	2nd inj., .1 cc intra-allantoic (1 hr later)	Hemagglutination titer* of allantoic fluids	Difference from controls, log
Saline	30 E.I.D.	224	
.4 mg PL†	"	2	2.15
Saline	30 E.I.D.	130	
.04 mg PL†	"	0	2.11
Saline	30 E.I.D.	65	
.01 mg PL†	"	6	1.06
.001 mg PL†	"	100	0
Saline	30 E.I.D.	103	
.04 mg PL‡	"	0	2.01
.01 mg PL‡	"	2	1.73
.001 mg PL‡	"	19	.75

* Expressed as reciprocal of geometric mean of hemagglutination titers of allantoic fluids from groups of 3 or 4 chick embryos obtained 6 days after inoculation.

† Polylysine of avg molecular wt 4900.

‡ Polylysine of molecular avg wt 20000.

to $10^{-4.30}$. It is therefore likely that in many cases the values for the reduction in virus concentration produced by polylysine, as represented by differences between treated and control hemagglutination titers, are minimum values due to the averaging into the mean of the zero values on the hemagglutination titer scale. To elucidate this, a separate experiment was done in which both hemagglutination and infectivity titers were simultaneously determined. A difference in hemagglutination titers between treated and control embryos was log 2.17, representing a 150-fold decrease in virus concentration (Table II). However, the actual reduction in virus concentration produced by polylysine, as revealed by infectivity titrations, was log 4.0, or a 10,000-fold decrease in virus production. Therefore the inhibition of mumps virus multiplication is probably greater than here reported in many instances where zero hemagglutination titers were obtained. These results also clearly demonstrate that the reduction in the hemagglutination titers of polylysine treated embryos was not due to an inhibition of the hemagglutination reaction, but represents a true inhibition of virus multiplication.

(b) *Effect of different quantities of polylysine on mumps virus multiplication.* In Table III are summarized the results of experiments to determine the least amount of polylysine which would inhibit mumps virus multiplication. Levels of polylysine from 1 to 40 μg were administered to embryos subsequently treated with mumps virus. Polylysine of molecular weight 4900 was effective in inhibiting virus multiplication at the 10 μg level but not at the 1 μg level. Polylysine of molecular weight 20,000 was even more effective, inhibition of virus multiplication be-

TABLE IV. Effect of Time Interval between Injections of Mumps Virus and Polylysine.

1st inj., .1 cc intra-allantoic	Interval, hr	2nd inj., .1 cc intra-allantoic	Hemagglutination[*] titer of allantoic fluids Treated	Hemagglutination[*] titer of allantoic fluids Control	Difference from controls, log
.4 mg PL[†]	1	30 E.I.D.	2	224	2.15
30 E.I.D.	8	.4 mg PL	0	130	2.11
”	24	.2 mg PL	1	204	2.16
”	36	.2 mg PL	0	204	2.31
”	44	”	24	46	.35
”	52	”	5	46	.98
”	56	”	106	65	0
”	80	”	96	65	0

[*] Expressed as reciprocal of geometric mean of hemagglutination titers of allantoic fluids from groups of 3 or 4 chick embryos obtained 6 days after inoculation with virus.

[†] Polylysine of avg molecular wt 4900.

TABLE V. Effect of Injection of Polylysine into Yolk Sac upon Hemagglutination Titer of Mumps Virus in Allantoic Fluid of Chick Embryos.

1st inj., .1 cc	Time interval, hr	2nd inj., .1 cc	Hemagglutination titer[*] of allantoic fluids	Difference from controls, log
Saline (AS)[†]	1	30 E.I.D. (AS)	204	
.4 mg PL[‖] (YS)[‡]	1	”	0	2.31
Saline (AS)	1	30 E.I.D. (AS)	100	
.2 mg PL[§] (YS)	26	”	32	.45
.2 mg PL[‖] (YS)	1	”	24	.62

[*] Expressed as reciprocal of geometric mean of hemagglutination titers of allantoic fluids from groups of 3 or 4 chick embryos obtained 6 days after inoculation.

[†] AS = Allantoic sac route.

[‡] YS = Yolk sac route.

[§] Polylysine of avg molecular wt 4900.

[‖] ” ” ” ” ” 20000.

ing obtained with only 1 μg of the polypeptide.

(c) *Effect of time interval between injection of mumps virus and polylysine.* Experiments were carried out to determine how long after inoculation of mumps virus polylysine was capable of causing inhibition of virus multiplication (Table IV). A single injection of 0.2 mg of polylysine, given 1 hour before or as late as 36 hours after inoculation of mumps virus, drastically inhibited viral multiplication. In all cases the differences between hemagglutination titers of allantoic fluids obtained from controls and those treated within 36 hours was over 2.0 log units. This reflects at least a 99.5% inhibition of viral multiplication. Inhibition by polylysine became less striking as the time interval was further increased, and no inhibition could be demonstrated when the polypeptide was inoculated 56 hours after infection with mumps.

(d) *Effect of injection of polylysine into yolk sac.* Experiments were carried out to determine whether injection of polylysine by a route other than the allantoic sac would also cause inhibition of mumps virus multiplication in the allantoic sac. The injection of 0.4 mg of polylysine, of molecular weight 4900, in the yolk sac 1 hour before inoculation of mumps virus in the allantoic sac completely inhibited hemagglutinin formation in the allantoic sac (Table V). However, polylysine of molecular weight 20,000 when injected in the yolk sac had much less inhibitory effect on the multiplication of mumps virus in the allantoic sac. It appears that the smaller polypeptide can more readily pass from the yolk sac to its site of action in the allantoic sac. When the smaller polypeptide was inoculated in the yolk sac 26 hours prior to injection of mumps virus, no significant inhibition of viral multiplication was obtained. Apparently, polylysine is metabolized, bound to protein, or removed in some manner from circulation within 26 hours after inoculation. This is supported by previous work which showed that polylysine cannot be demon-

TABLE VI. Effect of Polylysine on Mumps Virus *In Vitro.*

Mixture held at 3°C 30 min.		Constituents of inoculum			
Mumps virus dilution	Solvent	Mumps virus	PL, μg/embryo	Infectivity score	Virus titration end point ID_{50}, log
10^{-4}	Saline	10^{-6}	0	3/3	
”	”	10^{-7}	0	1/4	
”	”	10^{-8}	0	0/4	−6.75
10^{-4}	PL,* .5 mg/cc	10^{-6}	.5	3/3	
”	”	10^{-7}	.05	2/4	
”	”	10^{-8}	.005	0/4	−7.00

* Polylysine of avg molecular wt 4900.

strated in allantoic fluid by hemagglutination technic 24 hours after inoculation into the allantoic sac of chick embryos(9).

(e) *Effect of polylysine on mumps virus in vitro.* It was of interest to determine whether the very basic lysine polypeptide caused inactivation of the mumps virus particle. Hence, mumps virus was incubated with polylysine as indicated in Table VI, serial decimal dilutions of the mixture were then made with saline, and a virus infectivity titration carried out. As a control, the same procedure was repeated substituting saline for polylysine in the incubation mixture. Despite the presence of a high concentration of polylysine and a relatively low concentration of mumps virus in the incubation mixture, the infectivity titer of the virus after treatment with polylysine was not significantly different from the control, indicating that polylysine was not virucidal and that the effects observed *in vivo* cannot be readily explained by an inactivation of mumps virus by polylysine. However, these findings do not rule out the possibility of a reversible combination of polylysine with mumps virus in such manner as not appreciably to inactivate the virus.

Discussion. The evidence here presented demonstrates that polylysine inhibits multiplication of mumps virus in the allantoic sac of the chick embryo. These results are particularly striking since marked inhibition was obtained when large quantities of virus were given (*i.e.*, 300,000 infectious doses). Inhibition of viral growth was obtained with as little as 1 μg of polypeptide per embryo. It is important that the inoculation of polylysine as late as 36 hours after injection with mumps virus drastically inhibited viral multiplication, and that polylysine could be administered by a route other than that of virus inoculation; injection of a 4900 molecular lysine polypeptide in the yolk sac completely inhibited hemagglutinin formation in the allantoic sac.

Polylysine has been shown to be effective against a number of viruses in the chick embryo: influenza A, infectious bronchitis virus of chickens, Newcastle disease virus, mumps virus, and recently influenza B virus(10). The mechanism of action of polylysine in inhibiting viral multiplication deserves further consideration. The extreme reactivity of polylysine makes it difficult to discover precisely where in the chain of virus reproduction the polypeptide exerts its effect. It has been previously reported that polylysine will combine with red blood cells(9), tobacco mosaic virus(1), bacteria(11), nucleic acids(12) and many proteins(13). In view of this marked reactivity of the polypeptide, several possible modes of action of polylysine can be postulated: 1. The polypeptide might inactivate the virus particle *per se*. This is not supported by data here presented where it was shown that incubation of the virus with large quantities of polylysine did not demonstrably alter its infectivity. 2. Polylysine might combine with the virus particle extracellularly in a manner similar to neutralizing antibodies so that the virus cannot attach to the host cell. However, antibodies in general are completely ineffective in preventing viral proliferation once virus has penetrated the cell. In contrast, polylysine is effective when inoculated up to 36 hours after infection with mumps virus. Thus, polylysine inhibited viral multiplication when inoculated any time within the first cycle of multiplication and even into the second cycle of multiplication(14). In this respect, the action of polylysine in inhibiting viral multiplication is different from that of antibodies. 3. Polylysine might combine with receptor cell sites and thus prevent virus-cell interaction. This is suggested by the fact that both virus and polylysine combine with and agglutinate red blood cells. However, there is no necessary correlation between the ability of polypeptides to inhibit viral proliferation and to combine with red blood cells, for polyglutamic acid, which also agglutinates erythrocytes(10), does not inhibit viral multiplication. It is important in this respect to point out that polylysine is effective as an inhibitor of mumps virus multiplication in the chick embryo when inoculated after virus adsorption is complete. There remains a possibility that virus-cell interaction might be prevented in the second cycle of multiplication of the virus. However, since polylysine is not effective when given 26 hours before virus it would seem that the polypeptide would not remain available in the embryo over the 32 hours required for a complete cycle of multiplication of mumps virus.

4. Polylysine might prevent release of new virus particles from infected cells. We have no experimental data bearing on this possibility. 5. Polylysine might act intracellularly by diffusing into the cell and combining with intact virus, virus fragments, or intermediates in virus formation within the cell. Combination with intermediates in virus synthesis would seem possible in view of the known reactivity of polylysine and the ease with which it combines with proteins, nucleic acid, and tobacco mosaic virus by formation of ionic bonds with acidic groups. Such a combination should obey the law of mass action and an equilibrium would be established between polylysine, virus-intermediate and the polylysine-virus-intermediate complex. If this were the case, increasing the amount of polylysine administered would reduce the concentration of free virus intermediate and virus multiplication would be correspondingly diminished. Likewise by increasing the virus inoculum or by delaying the injection of polylysine for sufficient time, the concentration of free virus intermediate would be increased and inhibition reduced. The results reported here are consistent with this explanation.

Virus inhibition by lysine polypeptides may involve more than a single mode of action. The biological effects of the polylysine are no doubt associated with its reactivity in combining with acidic groups. Whether these groups involved are on the surface of virus particles, virus intermediates, virus receptor sites or other critical substances necessary for virus formation cannot yet be decided. It is possible that such combinations may occur outside the cells, on the cell surface, or within the infected cell.

The antiviral activity of the lysine polypeptides is sufficiently high to warrant further investigation. These studies are being continued and the effects in experimental animals are now under study.

Summary. Lysine polypeptides cause marked inhibition of the multiplication of mumps virus in the allantoic sac of the chick embryo. Inhibition was obtained with as little as 1 μg of polypeptide. Polylysine was effective when injected as late as 36 hours after inoculation of virus. The polypeptide caused inhibition of viral multiplication in the allantoic sac when injected either in the allantoic sac or the yolk sac. Possible mechanisms of inhibition of viral multiplication are discussed.

---

1. Stahmann, M. A., Graf, L. H., Patterson, E. L., Walker, J. C., and Watson, D. W., *J. Biol. Chem.*, 1951, v189, 45.

2. Rubini, J. R., Rasmussen, A. F., Jr., and Stahmann, M. A., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 662.

3. Watson, D. W., and Bloom, W. L., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 29.

4. Green, M., Rasmussen, A. F., Jr., and Stahmann, M. A., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 641.

5. Becker, R. R., and Stahmann, M. A., *J. Am. Chem. Soc.*, 1952, v74, 38.

6. Green, M., and Stahmann, M. A., *J. Biol. Chem*, 1952, v197, 771.

7. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

8. Ginsberg, H. S., Goebel, W. F., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, v87, 385.

9. Rubini, J. R., Stahmann, M. A., and Rasmussen, A. F., Jr., PROC. SOC. EXP. BIOL. AND MED, 1952, v76, 659.

10. Green, M., and Stahmann, M. A., unpublished data.

11. Burger, W. C., and Stahmann, M. A., *Arch.*

Biochem. Biophys., 1952, v39, 27

12. ———, *J. Biol. Chem.*, 1951, v193, 13.

13. Rice, R. V., Stahmann, M. A., and Alberty, R. A., unpublished data.

14. Ginsberg, H. S., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1949, v90, 393.

Received July 27, 1953. P.S.E.B.M., 1953, v83.

Thromboplastinase, a New Enzyme Which Destroys Thromboplastin. (20515)

S. GOLLUB, D. FELDMAN,* D. C. SCHECHTER, F. E. KAPLAN, AND D. R. MERANZE.
From the Department of Research, Albert Einstein Medical Center, Southern Division, Philadelphia.

The nature of thromboplastin has long been a subject of investigation. This material is clearly described only in terms of its activity: the initiation of the blood coagulation process. The most active, best characterized thromboplastic preparation to date is the lipoprotein fraction obtained by differential centrifugation according to Chargaff(1). Whether thromboplastic activity is associated with a specific molecule, a type molecule, or a combination of factors awaits clarification. The nature of the active lipid moiety of thromboplastic preparations likewise remains obscure. The relationship between the active materials in thromboplastic suspensions prepared from different tissues, as well as that found in human blood is undetermined. The preparation, preservation, variation in activity and fundamental mode of action of this material consequently remain empirical and ill-understood.

This laboratory recently reported on the marked reduction of thromboplastic potency in tissue suspensions due to a bacterial contaminant(2). The organism was later found to accomplish this potency reduction by virtue of an adaptive enzyme which it elaborated. Bacteriological studies are presented elsewhere (3).

This communication will describe the preparation and properties of the new enzyme, tentatively called "thromboplastinase" which we believe will be a useful tool in the investigation of thromboplastin. Our studies have led to evidence for a structural moiety necessary for thromboplastic action which is common to thromboplastic suspensions of different origins.

Materials. Glassware. All glassware was scrupulously cleaned in detergent, dichromate cleaning solution and then thoroughly rinsed. *Plasma.* Normal human plasma was collected in 1/9 volume oxalate in glass test tubes, centrifuged, and the supernatant plasma removed. *Thromboplastin.* Rabbit brain(4), human placenta(5), or human brain(3) were used as sources of thromboplastin. *Thromboplastinase.* An adaptive enzyme elaborated by a *Bacillus cereus-B. megatherium* intermediate under certain conditions.†‡ This organism grew well in a simple medium of salts and sugar, without elaborating thromboplastinase. However, if the substrate was a biological source medium such as a thromboplastic tissue extract or Brain Heart Infusion, (Difco), the enzyme was formed. Growth and enzyme production proceeded as well when the dialysate of Brain Heart Infusion was used as the medium. The dialysate was therefore employed to avoid the extraneous protein of the Brain Heart Infusion. The following method was then used to obtain crude enzyme.

A Brain Heart Infusion dialysate was prepared by placing a dialysis bag containing 250 ml of 0.9% Brain Heart Infusion into 1 liter of distilled water in a 2 liter flask and autoclaving at 15 lb for 20 minutes. Upon cooling, the sterile dialysate was seeded with a 24-hour (37°C) culture from a Brain Heart Infusion-agar slant and allowed to incubate at 37°C for 24 hours. During the course of this incubation, a progressive increase in enzyme activity of the culture medium was

---

* Present address: Laboratories, Brooklyn V. A. Hospital, Brooklyn, N. Y.

† Previously mistakenly reported to be *B. subtilis*.

‡ Obtainable from ATCC as No. 10987 (NR Smith's strain 248).

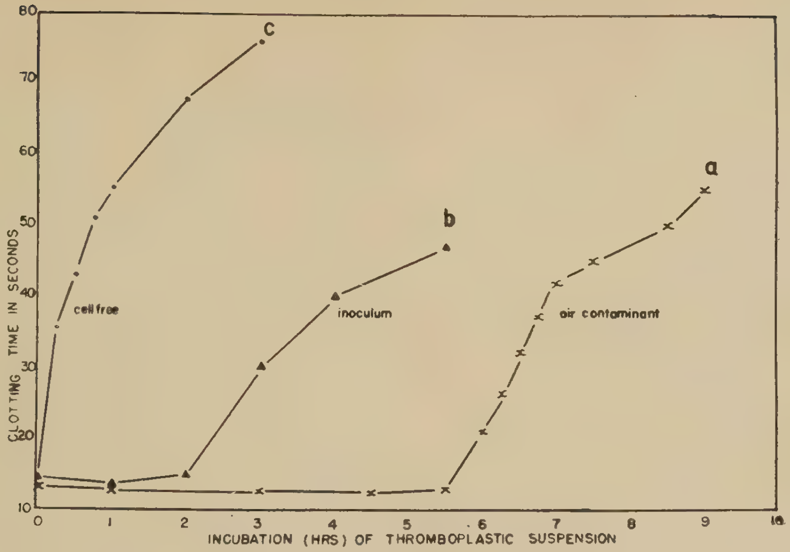


FIG. 1. Time course of thromboplastin destruction by thromboplastinase; a. from air contaminant; b. from an actively growing culture of the organism introduced as such into the thromboplastic suspension; c. from a cell-free crude enzyme preparation.

demonstrable. Incubation for longer periods, or at higher temperatures resulted in decreased activity or decreased elaboration of enzyme. The culture medium (1 liter), rendered cell-free by centrifugation,[§] was employed as a crude, active enzyme preparation. This crude cell-free preparation was stable for 2-3 days at 0-5°C. Partial purification and concentration of the enzyme preparation was effected by adsorption at 0-5°C on $Ca_3(PO_4)_2$ in final concentration of 0.2%. Elution at 25°C with 100 ml of 0.5 M Na Citrate was followed by centrifugation.

After dialysis at 0-5°C, which resulted in no loss of activity, this preparation was lyophilized. The yield was about 25 mg of a fluffy white powder which was stable indefinitely under refrigeration. Aqueous solutions of this powder were stable for a week under refrigeration.

Methods. Assay of enzyme activity: A modified one-stage test. The activity of the enzyme was measured in terms of substrate (thromboplastin) destruction. This was conveniently done by adapting the one stage method of Quick(4) to a test for thromboplastin.

When equal parts of normal human oxalated plasma, isotonic NaCl and $CaCl_2$ (.025 M) were mixed at 37°C and the clotting time determined, clot formation was observed after about 2 minutes. The reaction rate was greatly increased (the clotting time was sharply decreased) to about 13 seconds when a suspension of active tissue thromboplastin was substituted for the isotonic NaCl in the above procedure. Less active thromboplastic suspensions gave longer clotting times. The clotting time, as a function of the thromboplastic concentration, is shown in Fig. 2 where the various concentrations of thromboplastin, in percent, were obtained by simple dilution with saline of the thromboplastic stock preparation.

Thromboplastinase action was gauged then by the loss of potency of thromboplastic suspension after the enzyme and substrate were mixed and incubated. The incubating mixture consisted of 4 parts by volume thromboplastin, 5 parts $CaCl_2$ and one part thromboplastinase. The residual thromboplastic potency of the mixture was estimated by using it as a source of thromboplastin in the one-stage test. The calibration curve shown in Fig. 2 was used to translate to percent thromboplastin the clotting times obtained with the

---

[§] Seitz filtration is to be avoided as it causes a partial loss of enzyme activity by adsorption.

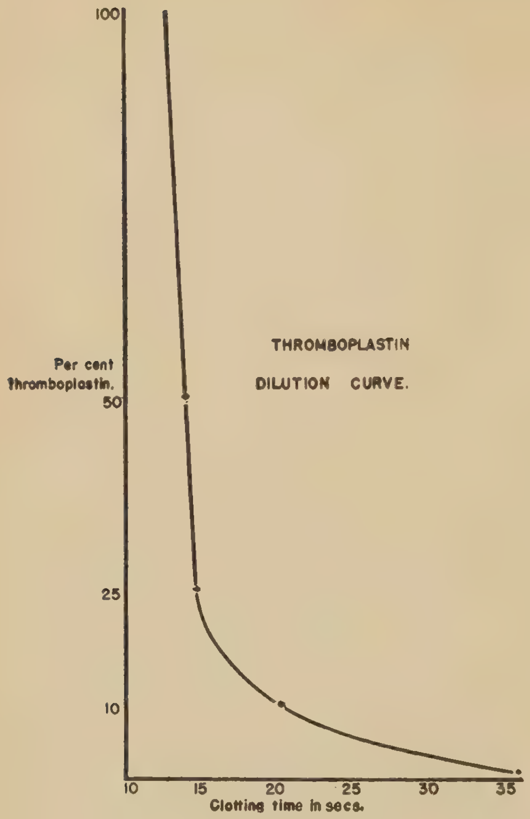


FIG. 2. Clotting time—dilution curve of an acetone-dried rabbit brain thromboplastin. 100% thromboplastin = least concentration which empirically yielded shortest clotting time.

incubated mixture.

To interpret the loss of thromboplastic activity as being due to destruction of thromboplastin, two conditions had to be satisfied: 1) boiled enzyme had to be ineffective and 2) no inhibitor should have been formed as a result of thromboplastinase activity. As will be seen below both these conditions were met. We therefore use Fig. 2 as a calibration curve for translation of clotting time to residual percent thromboplastin and by difference obtain the thromboplastin destroyed. Calibration curves should be set up for each laboratory, since thromboplastic potency is notoriously variable. Preparation of active, stable, reproducible thromboplastic suspensions has been described elsewhere(6).

Results. The time course of thromboplastic destruction by the bacterial enzyme thromboplastinase is shown in Fig. 1. Curve "a" represents that obtained when thromboplastic suspension is allowed to incubate in open vessels. The sudden rise of clotting time (loss of thromboplastic potency) was observed to occur after 6-8 hours. Plasma coagulation factors are not responsible for this clotting time rise(2).

Curve "b" represents that obtained when an actively growing culture of the organism is inoculated into the thromboplastic suspension. Here the "break" in the curve was seen to occur after about 2 hours.

Curve "c" is that obtained when a cell-free preparation of thromboplastinase was added to thromboplastic suspension. It is seen that thromboplastinase action was immediate.

The data summarized below indicate the enzymatic nature of thromboplastinase action. Fig. 3 shows that *the loss of thromboplastic potency caused by thromboplastinase was continuous over a time course.* Fig. 4 shows that *the rate of destruction of thromboplastin was a function of enzyme concentration. Small amounts of enzyme were effective*: using a crude preparation, less than 1 part of enzyme to 10,000 parts of thromboplastic suspension almost completely inactivated the latter in less than 5 minutes. *The active enzyme was recovered in the supernatant of the altered thromboplastic suspension.* In a typical experiment the enzyme concentration was adjusted so as to just give 90-100% inactivation of the thromboplastin in the given incubation time. Then employing high speed centrifugation (R.C.F. 20,000 G) the altered thromboplastic suspensions were separated from the supernatants containing the enzyme This supernatant was then used as the suspending fluid for fresh unaltered thromboplastic pellets obtained by similar centrifugation. Thromboplastic potency of this mixture was then determined after the same incubation period. Suitable controls were employed. The enzyme activity after 3 such recoveries was still high, approximately 98%, 92% and 83% thromboplastin being destroyed respectively.

The enzyme was inhibited by substances in trace amounts. The search for and use of inhibitors of thromboplastinase was complicated by the requirement that these inhibitors must not affect any of the reactions of the blood

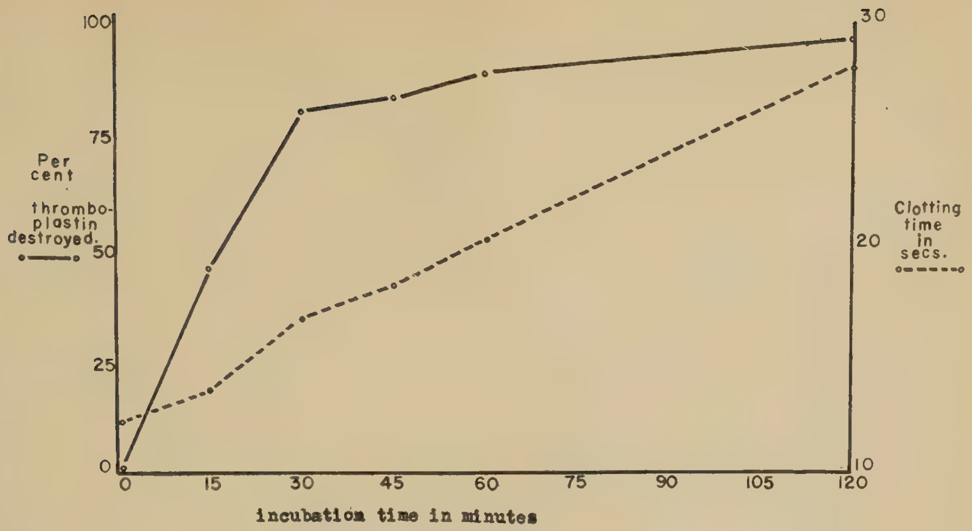


FIG. 3. Action of "thromboplastinase" on rabbit brain thromboplastin over a 120-min. time course. Boiled enzyme was ineffective.

clotting process since this is the means employed at present to assay thromboplastinase activity. Of many substances tested, cupric ion in final concentration of 10^{-4} was found to be suitable and to inhibit completely destruction of thromboplastin by thromboplastinase.

The action of thromboplastinase did not cause a pH change which might account for the loss of thromboplastic potency. When the reaction was allowed to proceed in the absence of added buffer, the pH of the incubating mixture remained at 6.4-6.5 throughout. Moreover, pH titration curves with acid or alkali pre- and post-incubation were not significantly different. *A clotting inhibitor was not formed by the action of the enzyme.* Neither the altered thromboplastic suspension, nor its centrifuged sediment, nor its supernatant showed thromboplastic activity or inhibition of thromboplastic activity when added to fresh thromboplastic suspensions. Thus the conversion of clotting time to percent thromboplastin discussed previously seems to be valid.

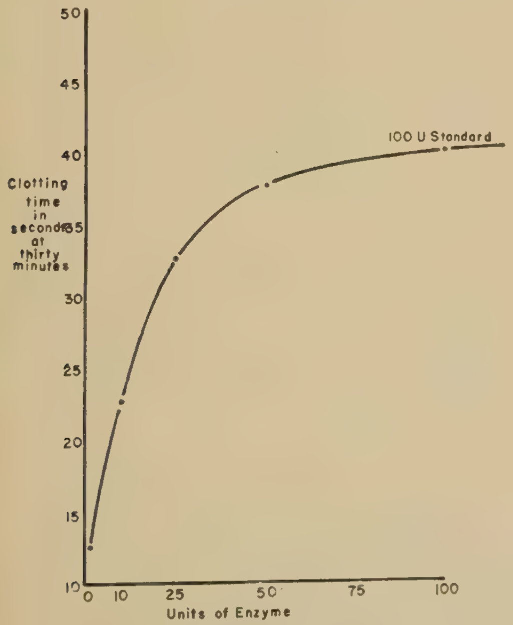


FIG. 4. "Thromboplastinase" activity as a function of concentration.

The data presented thus far were obtained with the enzyme acting on rabbit brain thromboplastic suspensions. In testing thromboplastinase against thromboplastic suspensions of other tissues and other animals, we found that thromboplastinase also decreased the potency of all thromboplastic suspensions tried to date (Table I). Note that Russel-Viper venom, a non-thromboplastic accelerator of blood clotting was not affected by thromboplastinase. The destructive action of thromboplastinase on thromboplastin of whole blood components is reported elsewhere(7).

We have, as yet, found no other enzyme to

TABLE I. Action of "Thromboplastinase" on a Variety of Thromboplastic Substrates.

Source	Clotting times (sec.) T zero	Clotting times (sec.) T 60 min.
Rabbit brain	12.6	50
" lung	11.4	82
Beef "	15.0	101
Human placenta	12.1	130
" brain	12.9	83
Rat "	27.9	65
Mouse "	41.1	95
Russel viper venom	20.2	21

cause such a rapid destruction of thromboplastin. Commercial preparations of lipase, rennin, papain, pepsin, trypsin, pancreatin and amylase did not reduce the clotting potency of thromboplastic suspensions. Crude jack bean meal urease caused a slight decrease in thromboplastin potency. Crystalline urease was without effect. Twelve organisms, common air contaminants, other than ATCC No. 10987, grown and tested under similar conditions failed to produce an active enzyme.

The action of the thromboplastinase is apparently highly specific. The enzyme yielded negative tests for lecithinase(8) phosphomonoesterase(9), lipase(10) ATP-ase(11) RNAse (12). Gelatin was not liquefied by the enzyme. No split of phosphorus was observed with cephalin as a substrate. There was no evidence of a nitrogen-split with thromboplastin as substrate using a micro-Kjeldahl procedure for analysis.

Attention was turned to the biochemical changes which bring about or accompany the loss of thromboplastic activity. The substrate employed in these investigations was a "Chargaff preparation" from human placenta. In these experiments, enzyme and substrate were mixed and incubated and aliquots removed over a time course for analysis. One aliquot was analyzed for thromboplastic potency; another was removed simultaneously, and after cold trichloracetic acid precipitation was centrifuged in the cold and analyzed for phosphorus content of the supernatant fluid. Three controls were employed: 1) An incubation mixture having the usual boiled enzyme control; 2) a control in which all factors were kept the same as the test incubation mixture except that copper was added initially to the incubation mixture and 3) a final control in which the copper was added after the reaction had been allowed to proceed to about half completion. These latter two controls were of special significance since it had been found, as mentioned earlier, that copper was an inhibitor of enzyme action. It was of interest then to determine whether chemical changes could be correlated with functional changes in thromboplastin. Results are shown in Fig. 5.

It is seen that with placenta thromboplastin as substrate, an increase in inorganic phosphorus of the supernatant was found, which was correlated with destruction of thromboplastin. The fact that addition of cupric ion to the reaction mixture either initially or during the reaction immediately stopped both loss of thromboplastic potency and the split of phosphorus suggests the identity of these two phenomena.

Further work of this type employing rabbit brain thromboplastin or human brain thromboplastin indicated that analyses for organic rather than inorganic phosphorus was a more reliable index of the chemical action of thromboplastinase. Such analyses were made by digesting the supernatant samples, then analysing for inorganic phosphorus by the method of King(13).

Discussion and conclusions. A previous

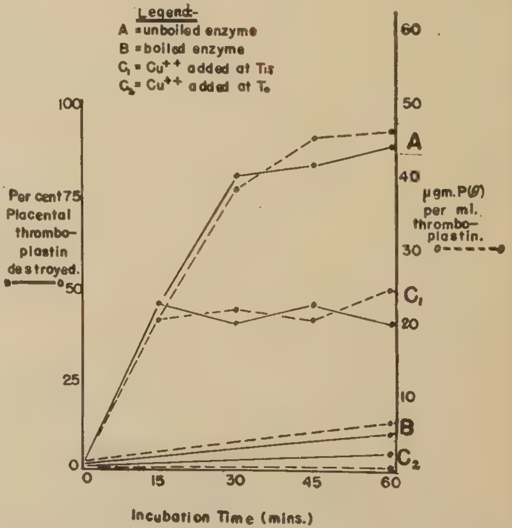


FIG. 5. Loss of thromboplastic activity and split of inorganic phosphorus due to enzyme action.

communication indicating a sudden potency loss of thromboplastic suspensions due to bacterial contamination can now be attributed to the elaboration of the enzyme thromboplastinase by a specific micro-organism. So far as we know, thromboplastinase is the only enzyme capable of destroying tissue thromboplastins of diverse sources, prepared in several different ways. The mode of action of this enzyme is apparently highly specific, involving a split of organic phosphorus seemingly correlated with the loss of thromboplastic potency. A conclusion from such evidence that tissue thromboplastin of different organs or of different species are the same is no doubt premature. It would appear, however, that they do contain a common structural unit, necessary for thromboplastic activity which is susceptible to thromboplastinase cleavage. Analysis of 3 different thromboplastic preparations indicates that this common moiety contains phosphorus. There seems, moreover, to be a quantitative relationship between thromboplastin destroyed and phosphorus liberated as a result of thromboplastinase action. This suggests the possibility of calibrating different thromboplastic preparations in terms of this functional phosphorus rather than in terms of non-specific nitrogen content of the tissue preparation. Further work reported elsewhere(14) indicates that the site of thromboplastinase action is on that portion of thromboplastic lipid which is associated with the inositol phosphatide fraction.

Summary. Thromboplastinase, a new enzyme of bacterial origin has been reported and its preparation and mode of action described. To our knowledge, this is the only enzyme which is apparently specific for thromboplastin. The split of a phosphorus moiety, apparently necessary for thromboplastic activity has been correlated with enzyme action. The theoretical and practical implications of these observations are discussed.

This work was supported in part by a grant, RG2927C, from the National Institutes of Health.

1. Chargaff, E., *J. Biol. Chem.*, 1944, v155, 387.
2. Gollub, S., Kaplan, F. E., and Meranze, D. R., Proc. Soc. Exp. Biol. and Med., 1950, v75, 725.
3. In preparation.
4. Gollub, S., Kaplan, F. E., and Meranze, D. R., *Am. J. Physiol.*, 1950, v162, 293.
5. Cohen, S. S., and Chargaff, E., *J. Biol. Chem.*, 1941, v140, 689.
6. Gollub, S., Kaplan, F. E., Meranze, D. R., and Schechter, D. C., *Science*, 1951, v114, 499.
7. Gollub, S., *Fed. Proc.*, 1953, v12, 54.
8. MacFarlane, M. B., and Knight, B. C. J. G., *Biochem. J.*, 1941, v35, 884.
9. Bodansky, A., *J. Biol. Chem.*, 1932, v99, 197.
10. Goldstein, N. P., Epstein, J. H., and Roe, J. H., *J. Lab. Clin. Med.*, 1948, v33, 1047.
11. Stern, K. G., Goldstein, G., and Albaum, H. G., *J. Biol. Chem.*, 1951, v188, 273.
12. Kunitz, M., and Simms, H. S., *J. Gen. Physiol.*, 1928, v11, 647.
13. King, E. J., *Biochem. J.*, 1932, v26, 292.
14. Feldman, D., Kaplan, F. E., and Meranze, D. R., *Fed. Proc.*, 1953, v12, 202.

Received July 27, 1953. P.S.E.B.M., 1953, v83.

Reaction of Certain Phytoagglutinins with Flexner-Jobling Carcinoma Cells of the Rat.* (20516)

W. J. Nungester and G. Van Halsema.

From the Department of Bacteriology, University of Michigan.

The complexity of the surface of all mammalian tissue cells is suggested by our knowledge of the various antigens that make up the surface of human erythrocytes. It is possible that the surfaces of cells vary not only with their histological classification, but also with their state of health. It is not illogical to postulate that the surface of epithelial cells of a carcinoma could be differentiated from the cell surfaces of related normal cells if proper

---

* Aided by a grant from the Michigan Memorial Phoenix Project.

means for making such measurements were available.

Several years ago the senior author and his colleagues(1) made a limited comparative study of the cell surfaces of guinea pig leukocytes and erythrocytes as measured by several different criteria, namely the reaction of these cells with 1) specific antiserums, 2) influenza virus, 3) pneumococcus polysaccharides, and 4) a phytoagglutinin. The results suggested that these or other biological substances might be selectively adsorbed to various mammalian cells. There is evidence that tumor antiserum and some viruses react with the surfaces of certain tumor cells. At least such substances will interfere with growth of such cells *in vivo*.

The work here reported comprises one portion of an investigation concerned with the determination of cell surface differences which may exist between cells obtained from normal and malignant tissues of the rat.

Phytoagglutinins have long been known to agglutinate various types of cells(2). More recently Boyd(3,4) has investigated numerous types of plant extracts for their hemagglutinating ability, looking especially for those plant substances which were able to specifically agglutinate cells of the different human blood groups. In the present study 5 different phytoagglutinins were used by the authors in a comparison of their action on rat leukocytes, erythrocytes, and tumor cells.

Materials and methods. The Flexner-Jobling carcinoma was used as a source of tumor cells. The tumor was maintained in white stock rats by the periodical subcutaneous inoculation of 0.5 ml of a 5% suspension of tumor cells. 12 to 15 day old subcutaneous tumors were aseptically excised, homogenized in a solution described by Krebs(5), filtered through 20XX mesh nylon, washed, and made up to the desired suspension on the basis of volume of packed cells.

White blood cells were obtained by the inoculation of 100 ml of Locke's solution containing 0.1% glucose into stock rats, and harvesting the peritoneal exudate approximately 12 hours later. The cells were spun down, washed, and resuspended to the desired concentration on the basis of volume of packed cells. Rat and chicken red blood cells were collected in citrate solution, washed, packed, and also resuspended to the desired concentration in saline (0.85% NaCl).

Four different phytoagglutinins were obtained from Dr. W. C. Boyd, of Boston, Massachusetts. These 4 lyophilized samples were reconstituted in saline solution so as to contain 10 mg per ml. A fifth phytoagglutinin was made from white navy beans after the method of Li and Osgood(6). The action of these phytoagglutinins against human red cells is as follows:

Phytoagglutinin No.	Source	Type of human erythrocyte agglutination
1.	*Phaseolus limensis* Macfadyen var. "Westside Lima"	A
2.	*Phaseolus vulgaris* Linnaeus var. "Golden Cluster Wax"	A, B, O
3.	*Phaseolus limensis* Macfadyen var. "Finast Lima"	A
4.	*Phaseolus vulgaris* Linnaeus var. "Rustproof Golden Wax"	A, B, O
5.	*Phaseolus vulgaris* Linnaeus var. "White Navy Bean"	A, B, O

Results. The agglutinating titers of the phytoagglutinins for the cells under consideration was determined using doubling serial dilutions of the stock solutions of the plant extracts made in saline. To each dilution an equal volume of 1% cells was added. The agglutination in each case was read after standing at room temperature for 2 hours. Chicken red blood cells were also tested for their agglutinability with these phytoagglutinins. The results of these tests are given in Table I.

From this table it is to be noted that phytoagglutinin numbers 2, 4, and 5 actively agglutinate rat erythrocytes, while only no. 5 agglutinates white blood cells, and no agglutination was observed with tumor cells. It was of further interest to determine whether or not the phytoagglutinins were removed from solution by the red cells that had been agglutinated, and also by the tumor cells which were not clumped by this material.

To determine this, the supernatant fluids of the tubes representing the end points in the agglutination reactions were assayed for the presence of remaining agglutinin. The supernatant from each series tested was di-

TABLE I. Agglutination Titers Obtained with Phytoagglutinins.

Phyto No.	Rat RBC	Rat WBC	Chicken RBC	Tumor cells
1	0	0	0	0
2	1:40	0	1:640	0
3	0	0	0	0
4	1:160	0	1:640	0
5	1:200	1:200	1:640	0

* 0 = no agglutination.

TABLE II. Tumor Growth Observed from Tumor Cells Treated with Unabsorbed Phytoagglutinins.

Phyto No.	10 days	15 days	20 days	25 days
1	4/10*	8/10	8/10	8/10
2	9/10	10/10	10/10	10/10
3	3/10	7/10	9/10	9/10
4	10/10	10/10	10/10	10/10
5	0/10	0/10	0/10	0/10
Control	10/10	10/10	10/10	10/10

* No. of tumor takes/No. of sites inoculated.

TABLE III. Tumor Growth Observed from Tumor Cells Treated with Adsorbed Phytoagglutinin No. 5.

Phyto No. 5	10 days	15 days	20 days	25 days
CRBC adsorbed	5/10*	6/10	6/10	6/10
Rat RBC adsorbed	5/10	10/10	9/10	9/10
Unadsorbed	1/10	1/10	1/10	1/10
Control	2/10	6/10	9/10	9/10

* No. of tumor takes/No. of sites inoculated.

vided into two portions, one to be titrated against rat erythrocytes and the other against chicken red blood cells. No agglutination of rat erythrocytes was obtained with the supernatant fluid following agglutination of rat erythrocytes by phytoagglutinins nos. 2, 4, or 5. However, these supernates did agglutinate chicken erythrocytes in a dilution of 1:640.

Using the supernates obtained from the first 2 dilutions in the tests with phytoagglutinins nos. 2, 4, and 5 on tumor cells, new agglutination tests with rat and chicken erythrocytes were also done. The rat erythrocytes were not agglutinated, indicating adsorption of phytoagglutinins 2, 4, and 5 by the rat carcinoma cells. Chicken erythrocytes were again agglutinated in a dilution of 1:640.

The results indicate that while agglutination occurs with rat erythrocytes and not with tumor cells, there is still evidence for the adsorption of the red blood cell agglutinin by both the red blood cells and tumor cells. On the other hand, the substance responsible for chicken red blood cell agglutination is not adsorbed by either rat erythrocytes or tumor cells.

Since the tumor cells obtained from the rat adsorbed phytoagglutinins 2, 4, and 5, it was desirable to ascertain whether this adsorption had any effect on the viability of the tumor cells. A known suspension of tumor cells was mixed with an equal volume of the stock solution of phytoagglutinin and placed on a shaker at room temperature for 2 hours, subsequent to which rats were inoculated subcutaneously with each mixture. Results as indicated by tumor growth are given in Table II. Phytoagglutinin no. 5 appears to have a marked inhibitory effect on tumor growth.

Phytoagglutinin no. 5 was adsorbed with chicken and rat erythrocytes in order to determine the inhibitory action of the adsorbed phytoagglutinin against tumor growth. A known tumor cell suspension was mixed with rat red blood cell adsorbed, chicken red blood cell adsorbed, and unadsorbed no. 5 phytoagglutinin, incubated at room temperature for 2 hours, and inoculated subcutaneously into rats. Results are given in Table III.

It appears that the rat red blood cell agglutinin is associated with the tumor inhibiting fraction, since after removal of the rat red blood cell agglutinin by adsorption, a greater incidence of tumor takes is noted.

Since the *in vitro* protective action of no. 5 agglutinin gave such significant results against tumor growth in rats, it was also of interest to determine the *in vivo* effect. Immediately after the inoculation of subcutaneous and intraperitoneal tumors, a defined amount of phytoagglutinin was injected intraperitoneally every day for a period of 10 days. In another group of rats, the same type of treatment was begun 5 days after tumor inoculation. In neither group did the treatment with phytoagglutinin *in vivo* have an inhibitory effect on tumor growth.

Summary. A phytoagglutinin obtained from the white navy bean, when added to a suspension of Flexner-Jobling rat carcinoma cells, destroyed the ability of these cells to produce tumors when inoculated into rats. The phytoagglutinin had no apparent effect *in vivo,* when administered daily to rats beginning at the time of the tumor inoculation.

1. Nungester, W. J., Gordon, Jean D., and Collins, Katherine E., *J. Infect. Dis.*, 1950, v87, 71.

2. Landsteiner, K., Raubitschek, H., *Centralblatt F. Bakt.*, 1907, v45, 660.

3. Boyd, W. C., Reguera, R. M., *J. Immunol.*, 1949, v62, 333.

4. Boyd, W. C., *J. Immunol.*, 1950, v65, 281.

5. Krebs, H. A., *Biochemica et Biophysica Acta*, 1950, v4, 249.

6. Li, G. J., and Osgood, E. E., *Blood*, 1949, v4, 670.

Received July 28, 1953. P.S.E.B.M., 1953, v83.

Development of Antibodies in Children Convalescent from Whooping Cough.* (20517)

Jeanette L. Winter. (Introduced by L. Emmett Holt, Jr.)

From the Department of Pediatrics, New York University College of Medicine and the Willard Parker Hospital, New York City.

The nature of the immunity which develops as a result of infection with *H. pertussis* has never been clearly defined. Many investigators believe that resistance to this disease is determined by the level of circulating antibacterial agglutinins. Evidence that this type of antibody frequently fails to appear as a result of the disease is, however, present in the literature. Other antibody responses which have been studied are complement fixation, opsonocytophagic reaction, and mouse protection. Recently Keogh and North(1) studied antihemagglutination and reported that in experimental murine pertussis the protective power of serum, given to mice intraperitoneally, was a function of the antihemagglutinin content of the serum—a conclusion not supported by the work to be reported here.

The present study is concerned with a comparison of 3 types of serum antibody in patients convalescent from pertussis, namely: 1) antibacterial agglutinin, 2) antihemagglutinin, and 3) mouse protective antibody (the capacity of serum to protect mice against intranasal infection with *H. pertussis*). It was the purpose of this study to ascertain which of these antibodies appeared most consistently with clinical recovery; hence which was the most significant measure of acquired immunity.

Methods and materials. Sera were obtained on patients ill with whooping cough from whom *H. pertussis* was isolated on nasopharyngeal culture.† The patients varied in age from 3 months to 9 years; their illnesses were characterized as moderately severe or severe. Blood was drawn immediately on admission and at 2- to 3-week intervals during convalescence. The sera were stored in the dry ice chest; all the specimens collected on each individual patient were tested together.

A. Antibacterial agglutinins. H. pertussis strain No. 18323 (obtained from Dr. Pearl Kendrick, Michigan Department of Health) and strain No. 484 (obtained from Dr. Anne Kimball, Minnesota Department of Health) were employed to prepare the standard antigen. The 24-hour growth from Bordet-Gengou Media was suspended in saline to a concentration of 20 billion organisms/ml, 0.3% neutral formaldehyde was added, and the bacterial suspension stored in the refrigerator. The antigen was used in a final concentration of 5 billion organisms/ml and was added to serial 2-fold dilutions in saline of the unknown serum. The tubes were shaken, and after 1-hour incubation at 37°C, the test was stored in the refrigerator overnight. Readings were made after 24 hours. Known positive and negative sera and antigen in saline were included as controls.

---

* This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service.

† These patients were studied at Willard Parker Hospital. We should like to express our thanks to the members of the house staff and nursing staff of the hospital for their cooperation in obtaining specimens for the study. In particular we are indebted to Dr. Zacharias Matthews, Dr. Doris Zenger and to Miss Wilma H. Penzes, R. N.

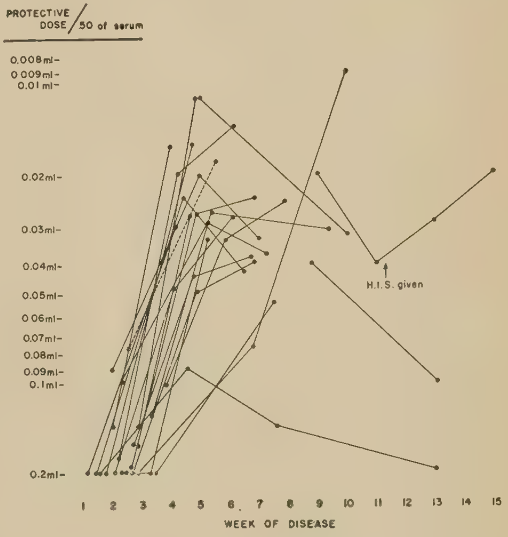
B. Antihemagglutinins. The method described by Keogh and North(1) was employed in the determination of antihemagglutinins, excepting that horse red blood cells were substituted for the human cells used in the original method; horse cells were found to be a more sensitive indicator of the presence of hemagglutinin. It was necessary however first to absorb out any horse cell agglutinins normally present in serum before testing for the presence of antihemagglutinin. The nonspecific lipoid hemagglutinin inhibitor present in many of the sera was extracted with acetone by the method of Chanock and Sabin(2). Supernatants of the 4-5-day growth in Cohen and Wheeler fluid media of *H. pertussis* strain No. 61 (obtained from Dr. E. V. Keogh, Department of Health, Commonwealth of Australia) served as the source of hemagglutinin in these tests. All dilutions were prepared in 0.02 molar phosphate buffer (pH 7), and readings were made by observing the pattern of the settled cells after the test had remained at room temperature for 1 hour.

C. The intranasal mouse protection test. The method of North, Keogh, Anderson, and Williams(3) was utilized in demonstrating mouse protective antibody. Swiss mice, Carworth Farms c.f.w. strain, 3-4 weeks old were injected intraperitoneally with 0.2 ml of graded dilutions of serum 4 hours prior to infection with the challenge culture. Serum dilutions were prepared in saline and ten mice were used for each dilution of serum; 0.2 ml of undiluted serum was the maximum amount tested.

Preparation of challenge: *H. pertussis* strain No. 18323 passed once through mouse brain and once through mouse lung was employed as the challenge strain. The LD/50 of the strain as determined by the intracerebral challenge(4) was 250 organisms before lung passage and 125 organisms after lung passage. Stock cultures of the reisolated strain were stored in the dry ice chest and a new vial was started for each test. The revived culture was grown on modified Bordet-Gengou media. The 24-hour growth from the 5th or 6th subculture on this media was used to prepare the challenge suspension. The growth was harvested into 1% casamino acids (pH 7-7.2) and standardized with the Klett photo-electric colorimeter to equal 10 billion organisms/ml. Further dilutions as needed were made in the 1% Casamino Acids.

Infective doses of 5 million, 50 million and 500 million organisms in 0.05 ml volume were used to determine the virulence of the culture. A dose of 50 million organisms in 0.05 ml volume (representing 2.5 to 5 LD/50 of the culture) was used as the standard infecting dose for testing the passive protective power of the unknown serum. This challenge dose regularly killed 80% to 100% of the control mice.

Intranasal inoculation was performed by the method of Burnet and Timmins(5). The mice were observed daily for 21 days; at the end of this period the PD/50 of the serum (the amount of serum needed to protect 50% of the mice against the 50 M challenge) and the LD/50 of the culture were calculated by the method of Reed and Muench(6). The acute and convalescent serum of each patient were assayed simultaneously. A standard



H.I.S.— Hyperimmune Serum.

FIG. 1. Development of mouse protective antibody in patients convalescing from pertussis. One patient [broken line (------)] had received 2 doses of vaccine prior to developing whooping cough.

TABLE I. Comparison of Mouse Protection with Other Serological Methods. Sera of children with whooping cough.

Patient	Age	Wk of disease	Antibacterial titer*	Antihemagglutinin titer*	Mouse protection,† PD/50 of serum
					ml
1. J.D.	4 mo	2	< 20	< 20	>.2
		4	20±	< 20	>.2
		6	40±	< 20	.033
2. V.Z.	4 "	3	< 20	< 20	>.2
		4	< 20	< 20	>.2
		8	< 20	< 20	.054
3. A.R.	5 "	2	< 20	< 20	>.2
		5	320	< 20	.016
4. M.R. I	8 "	3	< 20	< 20	>.2
		7	< 20	qns	.076
		10	< 20	80	.009
5. T.G.¶	9 "	3	640	< 20	.076
		6	320	80	.018
6. L.J.	12 "	3	< 20	< 20	.19
		5	< 20	< 20	.027
7. S.A.	14 "	3	< 20	< 20	.1
		6	320±	< 20	.029
		8	80	< 20	.037
8. P.A.	15 "	2	< 20	< 20	>.2
		5	< 20	< 20	.03
9. D.D.	20 "	3	< 20	< 20	.13
		5	< 20	< 20	.027
		9	< 20	< 20	.031
10. M.L.	2 yr	2	< 20	< 20	>.2
		5	< 20	< 20	.024
		7	< 20	< 20	.043
11. B.B.	2 "	2	40	< 20	>.2
		5	320	< 20	.09
		8	160	< 20	.14
		12-14	< 20	< 20	>.2
12. M.Q.	2 "	3	< 20	< 20	.158
		5	20±	< 20	.027
		7	40±	< 20	.024
13. R.R.	2 "	3	< 20	< 20	>.2
		5	< 20	< 20	.051
		7	< 20	< 20	.04
14. C.W.	2 "	2	< 20	< 20	>.2
		4	80	< 20	.049
		6	320	< 20	.028
15. L.R.	3 "	2	< 20	< 20	.14
		4	< 20	< 20	.016
16. M.D.	3 "	2	< 20	< 20	.18
		4	< 20	320±	.02
		6	< 20	320±	.014
17. M.R. II	6 "	3	< 20	80	.14
		5	< 20	160	.011
18. S.N.	6 "	3	< 20	< 20	.16
		5	< 20	< 20	.044
		7	< 20	< 20	.038
19. H.G.	8 "	3	< 20	80	.09
		6	80	320	.02
		8	40±	320	.033
20. V.H.	9 "	4	< 20	80	.1
		6	< 20	320	.033
		8	< 20	320	.025
Patients studied only during convalescence					
a. R.A.	2 yr	5	40	< 20	.011
		10	< 20	< 20	.032
b. G.P.	4 mo	9	40±	< 20	.04
		13	< 20	< 20	.1
c. S.S.	5 "	9	< 20	< 20	.02
		11	< 20	< 20	.04
		H.I.S.‡			
		13	80±	< 20	.029
		15	< 20	< 20	.02
Controls					
1. Normal infant serum			< 20	< 20	>.2
2. Human gamma globulin			< 20	< 20	>.2
3. Normal rabbit serum			< 20	< 20	>.2
4. Rabbit anti *H. influenza* serum			80±§	< 20	>.2
5. Rabbit *H. pertussis* immune serum			10, 240	< 20	.009
6. Human hyperimmune serum			320	40	.057 .045‖

* Reciprocal of titer.

† PD/50 = Amount of serum which protected 50% of the mice against intranasal challenge with 50 M. organisms of *H. pertussis* #18323.

‡ H.I.S. = Human hyperimmune serum administered.

§ Cross agglutination due to presence of agglutinins for *B. bronchisepticus*.

‖ Maximum and minimum PD/50 values obtained for this serum in 10 tests.

¶ Received two doses of pertussis vaccine prior to developing whooping cough.

serum of known protective potency was included in all the tests.

Results. A. The development of antibacterial agglutinins and antihemagglutinins during convalescence. In a group of 50 patients studied 31 or 62% developed antibacterial agglutinins, and only 7 or 14% developed measurable antihemagglutinins. Some of the patients who failed to show the development of antibacterial agglutinins when tested with the standard antigen were retested with antigen prepared from their own strain; again the results were negative. The demonstration of the antihemagglutinins was complicated by the presence in many sera of the non-specific lipoid inhibitor reported by Fisher and Keogh (7). With acetone extraction of the serum (method of Chanock and Sabin(2)) and controlled conditions of testing (Ludewig and Chanutin(8)) the interference of this inhibitor was avoided. It was observed that whereas many of the sera obtained during the acute phase contained non-specific inhibitor in high titer, it was usually absent from sera obtained during the convalescent stage of the disease.

B. Development of mouse protective antibody during convalescence. In twenty of the aforementioned patients mouse protective antibody titers were determined concomitantly with the antibacterial agglutinins and the antihemagglutinins. Fig. 1 and Table I show the results obtained in these patients and in 3 additional patients tested only during convalescence. Sera tested in the acute phase of the disease contained only minimal amounts of antibody. During convalescence mouse protective antibody titers increased in every case, the increases as a rule being from 4- to 10-fold. Some children reached their maximum concentration of protective antibody during the fifth or sixth week and subsequently showed some decline in titer, while others continued to show increases during the seventh to eighth week and later. In general the development of the antibody titers was parallel to the course of recovery. Of the three cases tested only during convalescence, two showed decreasing titers. The third child showed a decrease in titer associated with an increase in severity of her disease; human hyperimmune sera was given and there was a temporary improvement followed by another relapse; the patient finally recovered at a time when the mouse protective antibody titer had risen to its initial high level. The poorest response observed was in a 2-year-old child with a severe attack complicated by a protracted bronchopneumonia; in this child the maximum increase was only 2-fold and occurred in the fifth week of illness.

TABLE II. Comparison of Development of Mouse Protective Antibody with Other Serological Methods. Children with whooping cough.

Antibody measured	Total No. of patients studied	Patients showing rise in antibody titer: No. of patients	Patients showing rise in antibody titer: % of patients
Antibacterial agglutinins	50	31	62
Antihaemagglutinins	50	7	14
Mouse protective antibody	20	20	100

Table I compared the antibacterial agglutinins, antihemagglutinins, and the mouse protective antibody titers observed in each patient. Increases in mouse protective antibody occurred in patients who failed to develop measurable antibacterial agglutinins (12 patients), antihemagglutinins (14 patients), or both of these antibodies (8 patients). In cases where both the antibacterial agglutinin and mouse protective antibody developed the rise and fall of these 2 titers occurred simultaneously; no correlation could be made however between the magnitude of the respective titers. Antihemagglutinin was the most irregular in development.

The total number and the proportion of patients who developed each of the 3 antibodies studied is shown in Table II.

Discussion. The uniform development of mouse protective antibody during convalescence as demonstrated by the intranasal method of challenge suggests that this antibody may play an important role in immunity to the disease. The ability of patients to develop increases in mouse protection in the absence of antibacterial agglutinins and antihemagglutinins further supports this hypothesis. The lack of quantitative correlation between the mouse protective antibody and the antibacterial agglutinin titers found in the present study has been reported also by North *et al.* (3) in adult contacts and immunized children, by Mishulow *et al.*(9), and others. Similarly Hink and Johnson(10) working with mice given human hyperimmune plasma subsequently challenged by the intracerebral route found that the gamma$_2$ globulin contained approximately 90% of the mouse protective antibody and less than half of the Phase I agglutinins present in whole plasma.

It has not been possible to establish the relationship between antihemagglutinin and mouse protective antibody postulated by Keogh and North(1). The fact that only a small number of the patients developed measurable antihemagglutinins was unexpected and contrasted sharply to the ease with which this antibody could be produced in high titer as a result of vaccination of both human beings and laboratory animals with hemagglutinin containing fractions of *H. pertussis*. Although hemagglutinin is antigenic, additional evidence that the mouse protective power of serum is not determined by the antihemagglutinins has now been reported by Masry(11).

From limited data it would appear that a marked decline in circulating mouse protective antibody may occur within two or three months of the disease although some individuals have shown measurable titers when tested as long as 4 years after their illness. While circulating mouse protective antibody frequently disappears in the course of time, the immunity to whooping cough is known to persist. It is possible by means of a recall (booster) reaction to demonstrate a sharp increase in protective antibody in individuals with a known history of disease and in most adults(12). A corresponding increase in antibacterial agglutinins and antihemagglutinins may or may not occur. Whether this conditioned antibody response can explain the continued state of immunity is still undetermined. Further work along these lines is being carried out.

The mouse protection test is being applied to a study of children immunized against whooping cough as an important tool in the evaluation of different methods of vaccination against this disease.

Summary. 1. In a study of the development of antibacterial agglutinin, antihemagglutinin, and mouse protective antibody in children convalescing from whooping cough, mouse protective antibody was the only one to occur regularly as a result of the disease; it appears to be the most significant measure of immunity studied. 2. Mouse protective antibody titers increased during convalescence in all instances, the increases generally being from 4- to 10-fold. 3. The intranasal mouse protection test has been found to give reproducible results and is being applied to the study of children immunized against whooping cough.

We gratefully acknowledge the very valuable help and the encouragement given throughout this study by Dr. Robert Ward and Dr. L. Emmett Holt, Jr. Thanks are also due Mr. Clarence Johnson for his technical assistance.

---

1. Keogh, E. V., and North, E. A., *Australian J. Exp. Biol. and Med. Sc.*, 1948, v26, 315.

2. Chanock, R. M., and Sabin, A. B., *J. Immunol.*, 1953, v70, 302.

3. North, E. A., Keogh, E. V., Anderson, E., and Williams, S., *Australian J. Exp. Biol. and Med. Sc.*, 1939, v17, 275.

4. Kendrick, P. L., Eldring, G., Dixon, M. K., and Mesner, J., *Am. J. Pub. Health*, 1947, v37, 803.

5. Burnet, F. M., and Timmins, C., *Brit. J. Exp. Path.*, 1937, v18, 83.

6. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

7. Fisher, S., and Keogh, W. V., *Nature*, 1950, v165, 248.

8. Ludwig, S., and Chanutin, A., *J. Biol. Chem.*, 1949, v179, 271.

9. Mishulow, L., Siegel, M., Leifer, L., and Berkey, S. R., *Am. J. Dis. Child.*, 1942, v63, 875.

10. Hink, S., Jr., and Johnson, F., *J. Immunol.*, 1950, v64, 39.

11. Masry, F. L. G., *J. Gen. Microbiol.*, 1952, v7, 201.

12. Winter, J. L., Unpublished data.

Received July 28, 1953. P.S.E.B.M., 1953, v83.

Estimation of Total Body Chloride in Young Infants by Radiobromide Dilution.* (20518)

GILBERT B. FORBES,† ALLEN REID, JUNE BONDURANT, AND JOHN ETHERIDGE.

From the Departments of Pediatrics and Biophysics, Southwestern Medical School of the University of Texas, Dallas.

A number of recent reports have indicated the usefulness of bromine as a "tracer" for chlorine in isotopic dilution experiments, for the radio-isotopes of chlorine possess inherent disadvantages. Earlier studies indicated that the bromide ion is distributed in body fluids and tissues of animals in proportion to chloride with the exception of brain and cerebrospinal fluid(1,2). More recently Gamble and associates have found that the volumes of distribution of Cl^{38} and Br^{82} are quite similar in man(3).

Values for total body chloride in adults have been established at an average of 31.6 meq/kg body weight for males and 28.6 meq/kg for females(4). This study of total body chloride in newborn infants was undertaken for the purpose of establishing normal values in this age group and for comparison with data obtained by others by direct carcass analysis.

Methods. Radiobromine^{82}, obtained from Oak Ridge National Laboratories, was administered intravenously in doses approximating 2 μc/kg body weight according to the method of Barnett and Fellers(5). An equilibration period or 24 hours was employed, during which the subjects consumed their usual milk formulas and fluids and the urine was collected quantitatively. The subjects were normal infants 2 to 16 days of age. They were weighed at the beginning and end of the equilibration period and the average value used for purposes of calculation. Total body chloride was computed as follows:

$$\text{Total body Cl} = \frac{Br^{82}\text{ administered} - Br^{82}\text{ excreted}}{\text{Serum } Br^{82}/\text{serum Cl}}$$

Serum chloride was determined by a micro-modification of the method of Van Slyke and Hiller(6). Radioactivity measurements were made with an end-window Geiger counter with appropriate corrections for self-absorption, resolving time and radioactive decay.

TABLE I. Total Body Chloride in a Series of Infants.

Age, days	Sex	Wt, g	Height, cm	Total Cl, meq	meq/kg
2	♂	2095	44	133.2	63.6
10	♂	2140	44.5	126.0	58.9
16	♂	2255	42	113.1	50.2
6	♂	2325	46	117.1	50.3
2	♀	2395	44	126.5	52.8
5	♂	2480	49	119.2	48.0
12	♀	2545	50	104.9	41.2
6	♀	3070	50	134.4	43.8
					Avg = 51.1

Results. Results of determination of total body chloride in 8 normal infants are tabulated in Table I, with the subjects arranged according to weight. The average value of 51.1 meq/kg body weight is considerably greater than that reported for adults using comparable methods. Despite the rather wide range of the values, in none of our subjects did the value approach those noted for adult subjects(4).

Comparison with carcass analysis. Whereas no information is available on total body chloride as determined by direct whole body chemical analysis in adults, a number of newborn infants have been studied by earlier workers. These observations can be used to test the validity of the bromide dilution method as a measure of total body chloride. A total of 11 carcass analyses have been reported for subjects in the same general weight range as those reported in this paper, the average value for total body chloride being 49.2 meq/kg with a range of 40.9 to 54.4 meq/kg(7-11). This value and range of values are strikingly similar to those obtained by bromide dilution, and offer considerable support to the hypothesis that the method is

---

* Supported by a grant from the Playtex Park Research Institute.

† Present address: University of Rochester School of Medicine and Dentistry.

valid for young infants.

On theoretical grounds, one would expect the isotopic dilution values to be somewhat less than those obtained by carcass analysis since bromide^{82} equilibration in brain and cerebrospinal fluid is not complete at the end of 24 hours(12), yet precise data with which to evaluate the error involved are lacking. On the other hand, total body chloride progressively decreases as body weight increases during growth(13) and since the average weight of our subjects is approximately 480 g less than that of the subjects quoted above the 2 groups are not strictly comparable. According to equations developed by one of us (G.B.F.) from published data on carcass analysis it can be predicted that the average total body chloride for our group of subjects should have been 53.2 meq/kg, or 2.1 meq/kg more than the values observed.

Conclusion. 1.) Values for total body chloride, as determined by radiobromine^{83} dilution, averaged 51.1 meq/kg body weight for a series of normal newly born infants. Total body chloride, on a per kilogram basis is far higher in young infants than adults. 2.) Values obtained by this method show excellent agreement with those obtained by earlier workers employing direct chemical analysis. Therefore, the bromide dilution method would appear to be valid for the measurement of total body chloride in young infants.

1. Wallace, G. B., and Brodie, B. B., *J. Pharm. Exp. Therap.*, 1939, v65, 214; *ibid*, v65, 220.

2. Weir, E. C., and Hastings, A. B., *J. Biol. Chem.*, 1939, v129, 547.

3. Gamble, J. L., Jr., Robertson, J. S., Hannigan, C. A., Foster, C. G., and Farr, L. E., *J. Clin. Invest.*, 1953, v32, 483.

4. Dunning, M. F., Steele, J. N., and Berger, E. Y., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 854.

5. Barnett, H. L., and Fellers, F. X., *Science*, 1947, v106, 401.

6. Van Slyke, D. D., and Hiller, A., *J. Biol. Chem.*, 1947, v167, 107.

7. Camerer, W., and Soldner, *Z. Biol.*, 1900, v21, 173; *ibid*, 1902, v25, 1.

8. Hugounenq, L., *J. Physiol., Path. gén.*, 1900, v2, 509.

9. Michel, C., *Compt. rend. Soc. Biol.*, 1899, v51, 422.

10. Iob, Vivienne and Swanson, W. W., *Am. J. Dis. Child.*, 1934, v47, 302.

11. de Lange, C., *Z. Biol.*, 1900, v22, 526.

12. Present Authors, unpublished observations.

13. Forbes, G. B., *J. Ped.*, 1952, v41, 202.

Received July 28, 1953. P.S.E.B.M., 1953, v83.

Effects of Cortisone on Thiamine-Deficient Young Rats.* (20519)

A. M. WILWERTH AND JOSEPH MEITES.†

From the Department of Physiology and Pharmacology, Michigan State College, East Lansing, Mich.

Large doses of cortisone may increase requirements for vit. B_{12} and other factors in the young rat(1-3) and baby pig(4). Preexistent deficiencies of vit. B_{12} in these species were aggravated by cortisone injections, resulting in reduced body growth and survival rate. These detrimental actions of cortisone were largely counteracted in young rats by increasing vit. B_{12} intake at least 10 times above normal requirements (to 200 μg per kilo of diet). Addition of small amounts of aureomycin to the diet further enhanced the beneficial effects of vit. B_{12}, and both supplements were accompanied by increases in food intake and greater efficiency in converting food into body weight gains. It was the purpose of the present study to determine the effects of large doses of cortisone on requirements for thiamine by the young rat.

Methods and results. Exp. 1. Fifty-three young male Carworth rats were divided into

---

* Published with the approval of the Director of the Michigan Agric. Exp. Station as Journal Article No. 1541.

† These studies have been aided in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

TABLE I. Effects of Cortisone and/or Thiamine on Body Weight, Food Intake and Efficiency of Food Utilization.

Group and No. of rats	Treatment	Avg body wt, g Initial*	Avg body wt, g Final	Avg food intake, g Total	Avg food intake, g Per g gain body wt
	1st exp.—18 days duration				
1 (13)	1 mg thiamine	101.7 ± 2.3 †	151.5 ± 4.1†	153	3.0
2 (10)	No thiamine, no cortisone	105.6 ± 2.07	80.8 ± 3.1	65	—
3 (10)	Cortisone, no thiamine	105.2 ± 2.3	64.8 ± 2.9	62	—
4 (10)	" + 1 mg thiamine	102 ± 2.7	110.8 ± 5.1	135	15.3
5 (10)	" + 10 mg "	101.8 ± 2.8	137.6 ± 4.9	170	4.7
	2nd exp.—12 days duration				
1 (9)	1 mg thiamine	77.3 ± 1.1	107.3 ± 3.8	82	2.73
2 (9)	No thiamine, no cortisone	74.8 ± 1.8	62.0 ± 2.4	30	—
3 (10)	1 mg cortisone, no thiamine	73.0 ± 1.2	—	—	—
4 (10)	1 mg cortisone + 20 mg thiamine, pair-fed to group 3	73.8 ± 1.7	63.0 ± 1.4	30	—
5 (10)	1 mg cortisone + 1 mg thiamine	72.0 ± 1.7	71.2 ± 1.6	61	—
6 (9)	1 " " + 5 " "	75.5 ± 1.6	99.0 ± 6.3	96	4.1
7 (9)	1 " " +10 " "	73.1 ± 2.03	94.0 ± 3.6	93	4.4
8 (9)	1 " " +20 " "	71.3 ± 1.5	97.7 ± 2.1	105	4.0

* Represents avg body wt after thiamine-depletion period.
† Stand. error of mean.

5 uniform groups by weight and were fed the following semi-synthetic ration from which thiamine was omitted: alcohol washed casein, 25 g;[‡] glucose (Cerelose), 62 g;[§] corn oil (Mazola), 10 g; salt mixture, 4 g.[‖] To each kilo of the above the following vitamins were added: riboflavin, 5.0 mg; pyridoxine, 2.0 mg; Ca pantothenate, 28.0 mg; niacin, 10.0 mg; choline, 1 g; 2-Me-1, 4 Naphthoquinone, 0.4 mg; vit. A, 100,000 I.U.; vit. D, 125,000 I.U.[¶] After a depletion period of 10 days on this diet, each of the 5 groups was treated as shown in the upper part of Table I for a period of 18 days. Thiamine was incorporated into the ration and cortisone acetate was injected subcutaneously in 1 mg doses once daily. Measurements of individual body weight and average food consumption were made every 2 days. All rats were housed in metal cages with raised screen bottoms at a temperature of 78 ± 1.0°F.

The pertinent data are summarized in Table I, and the character of body growth and food intake are shown in Fig. 1. It can be seen that growth and appetite were reduced in all rats by the end of the 10-day depletion period. After this period, the rats fed 1 mg of thiamine per kilo of diet (Group 1) increased in average body weight from 101.7 ± 2.3 g to 151.5 ± 4.1 g and ate more food. The thiamine-deficient rats (Group 2) lost an average of about 25 g in body weight and ate less than half the food consumed by the rats in Group 1. An even greater loss in body weight occurred in the thiamine-deficient rats given cortisone (Group 3), although appetite was not depressed below that of the rats in Group 2. These rats were in the poorest state of any in the experiment and it is believed they would have died if cortisone treatment had been continued. In addition to extreme emaciation, they exhibited typical symptoms of thiamine deficiency such as hunched posture, paralysis and spinning when picked up by the tail. Eight of the 10 rats in this group showed pronounced priapism, a phenomenon not seen in any of the other rats. The addition of 1 mg of thiamine per kilo of diet enabled cortisone-treated rats (Group 4) to overcome partially the depression of body growth and eliminated other gross symptoms of thiamine deficiency. When 10 mg

---

[‡] "Vitamin Test" casein, Nutritional Biochemical Corp., Cleveland, O.

[§] Corn Products Refining Co., Chicago, Ill.

[‖] Salt mixture No. 2, Nutritional Biochemical Corp., Cleveland, O.

[¶] The B vitamins and cortisone acetate (Cortone) were supplied through the kindness of Dr. L. Michaud of Merck and Co., Rahway, N. J.

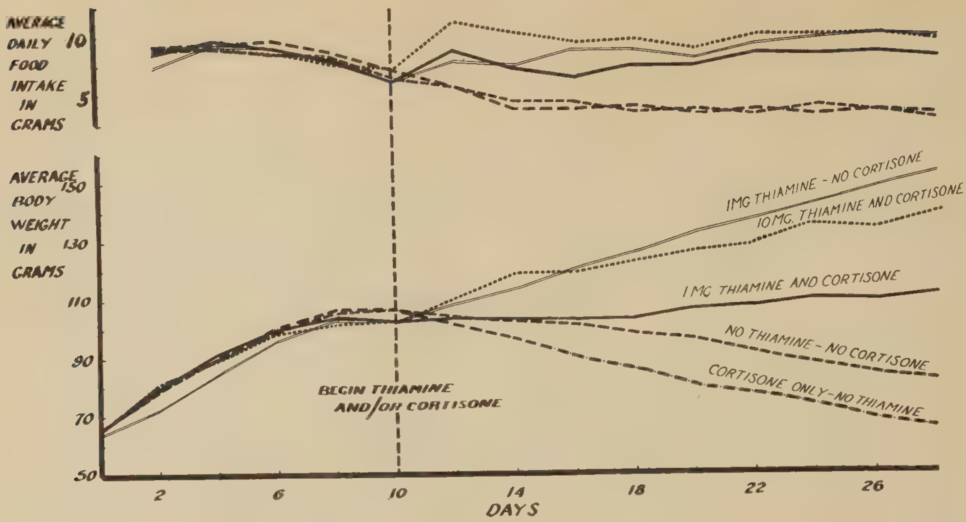


FIG. 1. Graph showing avg body wt and food intake of rats during 10-day, thiamine-depletion period and after treatment with cortisone and/or thiamine.

of thiamine per kilo were fed to cortisone-injected rats (Group 5), there was an almost complete counteraction of growth inhibition.

Exp. 2. A total of 75 young male Carworth rats were divided into 8 uniform groups by weight, and fed the thiamine-free ration for 18 days. At the end of this period each group was treated with thiamine and/or cortisone for 12 days as indicated on the bottom-half of Table I. The pertinent results are also given in this table.

The initial body weights of all rats averaged 46.7 ± 0.8 g, and at the end of the 18-day depletion period, 73.8 ± 1.6 g. The group which subsequently received 1 mg of thiamine per kilo of diet (Group 1) gained approximately 30 g each in body weight, while the animals which continued on the deficient diet (Group 2) lost about 12 g each in body weight. When cortisone was given to the thiamine-deficient rats (Group 3), a severe cannibalism developed which resulted in the death of 8 out of 9 rats by the end of 12 days. Priapism also appeared in most of these rats. The rats in Group 4 received cortisone and 20 mg of thiamine per kilo of diet, but were allowed only as much food as consumed daily by the rats in Group 3. Although these rats were reduced in body weight, they all survived and showed no gross symptoms of thiamine deficiency. When 1 mg of thiamine was fed to cortisone-treated rats (Group 5), there was no change in body weight but gross symptoms of thiamine deficiency were less marked than in Group 2. Groups 6, 7 and 8, received 5, 10 and 20 mg of thiamine per kilo of diet, respectively, and all levels were equally effective in counteracting the cortisone-induced inhibition of body growth and appetite. In this as well as in the first experiment, cortisone reduced the efficiency of transforming food into body weight gains.

Discussion. These data are believed to demonstrate that injections of large doses of cortisone can aggravate a pre-existent deficiency of thiamine in young rats, as shown by reduced body growth and survival rate and earlier manifestation of other extreme deficiency symptoms. The priapism, seen only in the cortisone-treated, thiamine-deficient rats, had previously been observed in young rats given huge doses of cortisone(5). The "toxic" effects of cortisone were more marked in rats of the 2nd than in those of the first experiment, presumably because they were younger and were initially made more deficient in thiamine prior to cortisone administration. Since 1 mg of thiamine per kilo of diet represents normal requirements for growth in young rats(6), the present data suggest that at least a 5-fold increase in thiamine is necessary to counteract growth-inhibition induced by injections of 1 mg of cortisone daily. These data also suggest that large amounts of thiamine have beneficial effects on cortisone-treated rats, apart from any effect on appetite. Rats given cortisone and thiamine, but limited in food intake to that of cortisone-injected, thiamine-deficient rats, at least survived and failed to show typical deficiency symptoms. Data reported by Schultz *et al.*(7) suggest that large doses of cortisone may also aggravate a pre-existent deficiency of pantothenic acid in rats.

Summary. (1) A total of 128 young male albino rats were used in 2 experiments to determine the effects of large doses of cortisone on thiamine requirements. They were fed a semi-synthetic ration from which thiamine was omitted for preliminary periods of either 10 or 18 days, followed by injections of 1 mg of cortisone daily with or without several levels of thiamine for another 18 or 12 days. (2) Pre-existent deficiency symptoms were aggravated by cortisone, resulting in decreased body growth and survival rate, priapism, cannibalism and loss of muscular coordination. The rats which were initially deficient in thiamine for the longer period, showed the most severe reactions to cortisone administration. Cortisone decreased the efficiency of converting food into body weight gains in all cases. (3) When 1 mg of thiamine per kilo of ration was fed, representing normal requirements for growing rats, there was little or no increase in body weight although most deficiency symptoms disappeared. When 5, 10 or 20 mg of thiamine per kilo of ration were fed, each level was equally effective in counteracting growth inhibition by cortisone.

---

1. Meites, J., Proc. Soc. Exp. Biol. and Med., 1951, v78, 692.

2. ———, *Metabolism*, 1952, v1, 58.

3. ———, Proc. Soc. Exp. Biol. and Med., 1952, v81, 307.

4. Wahlstrom, R. C., and Johnson, B. C., Proc. Soc. Exp. Biol. and Med., 1951, v78, 112.

5. Ershoff, B. H., Proc. Soc. Exp. Biol. and Med., 1951, v78, 836.

6. Brown, R. A., and Sturtevant, M., *Vitamins and Hormones*, 1949, v7, 176.

7. Schultz, R. B., Winters, R. W., and Krehl, W. A., *Endocrinology*, 1952, v51, 336.

Received July 28, 1953. P.S.E.B.M., 1953, v83.

Calorigenic and Antigoitrogenic Actions of L-Triiodothyronine and L-Thyroxine in Thyroidectomized and Intact Rats.* (20520)

Arthur E. Heming and Dorsey E. Holtkamp.

From the Research and Development Division, Smith, Kline and French Laboratories, Philadelphia.

3:5:3′-L-triiodothyronine has been reported to be 3 to 4 times as effective as L-thyroxine in preventing goitre development in thiouracil-treated rats(1). Also, in the treatment of 2 myxedematous patients, it appeared to be effective at doses lower than those required with thyroxine administration(2). On the contrary, Gemmill(3), using a small number of intact rats, has recently reported that the two agents produce quite similar calorigenic responses. This latter apparent discrepancy might possibly be the result of variability in the individual animal or strain, differences in experimental approach or in the type of animal preparation (intact or thyroidectomized) used. To further an understanding of these dissimilarities of response, a comparison has been made in intact and thyroidectomized rats with reference to calorigenic and antigoitrogenic potencies of these two agents.

Experimental. Male rats (Albino Farms), weighing between 125 and 175 g at the beginning of the experiment, were used. Purina Dog Chow Checkers and tap water were given *ad libitum* unless otherwise indicated. Both the triiodothyronine (3:5:3′-L-triiodothyronine) and thyroxine (L-thyroxine sodium pentahydrate) were given by subcutaneous injec-

---

* Presented in part at the Federation Meetings, Chicago, April 1953.

tion in a vehicle of 0.9% saline adjusted to pH 8.5. The concentration was such that each rat received 1 ml solution/kg/injection.

The rate of oxygen consumption of the individual rats was determined following a 24-hour fast, by measuring the oxygen consumed by each rat during three consecutive 15-minute periods. The expired carbon dioxide was collected over soda lime. Mean oxygen consumption of the three 15-minute periods was calculated, corrected to standard temperature and pressure, and converted to l/sqm/hr utilizing the Hill and Hill(4) formula of wt $^{2/3}$ x 10.

Thyroidectomies were performed surgically using ether anesthesia. Following surgery, the thyroidectomized rats were maintained on 1% calcium gluconate as the drinking fluid continuously, including the 24-hour fasting periods which preceded the oxygen consumption measurements. At least 30 days intervened between thyroidectomy and the first testing period. To assure completeness of extirpation, only those rats with an oxygen consumption of less than 5.5 l/sqm/hr were used. The pre-treatment mean oxygen consumption of the thyroidectomized rats was 4.7 l/sqm/hr (range 4.1 to 5.4 l/sqm/hr).

In the antigoitrogenic test, there were 8 groups of rats. One of these 8 groups served as a normal control and received tap water as the drinking fluid, while the other 7 groups received 0.1% thiouracil. Three of these 7 groups received daily injections of 1.25, 2.5, and 5.0 γ/kg/day of L-triiodothyronine, respectively; another 3 groups received 6, 12, and 24 γ/kg/day of L-thyroxine sodium pentahydrate, respectively; and the one remaining, which served as thiouracil-treated control, received injections of only the alkaline saline vehicle. Following 10 days of such treatment, the rats were sacrificed, and the thyroid glands were removed and weighed.

The calorigenic test in thyroidectomized rats consisted of measuring the response to these two compounds: 1) following chronic daily administration, and 2) after a single injection. For the former, 6 groups of 5 or 6 rats each were observed concurrently. One group was composed of intact control rats, and the other 5 groups consisted of thyroidectomized rats. One of these 5 groups remained untreated and served as the thyroidectomized control group. Following initial control observations, 4 of the 5 thyroidectomized groups were injected daily with 6, 12, 24, and 60 γ/kg/day of L-thyroxine sodium pentahydrate, respectively, for a 17-day period. This was followed, first, by a 17-day recovery period during which no drug was administered, and then a 17-day period of L-triiodothyronine administration at doses of 1.25, 2.5, 5.0 and 12.5 γ/kg/day respectively. The molar ratio between the respective doses of L-triiodothyronine and L-thyroxine sodium pentahydrate was maintained at 1:3.5 throughout all of these experiments. This ratio was chosen on the basis of the suggested activity ratio reported by Gross and Pitt-Rivers(1).

In studying the response to a single injection, 3 groups of 5 or 6 thyroidectomized rats per group were injected simultaneously at zero time with 1500 γ/kg of L-thyroxine sodium pentahydrate. Each group was observed at two different intervals during the ensuing 100-hr period so that the response was observed at about 4, 8, 24, 52, 75 and 98 hours after the time of administration of the drug. Nine days after the thyroxine administration, the rats were given a single injection of 312 γ/kg of L-triiodothyronine and tested at intervals similar to those following the thyroxine administration. The doses of both of these agents were 25 times larger than the highest doses used in the previously described study involving chronic daily administrations.

For the study in intact, non-thyroidectomized rats, a somewhat different procedure was employed. Here, the rats were injected for 3 days and the rate of oxygen consumption was determined on the fourth day. In this case, 4 groups of 6 rats each were observed concurrently. One was treated with only the alkaline saline vehicle, one with 0.1 mg/kg/day of L-thyroxine sodium pentahydrate, one with 0.073 mg/kg/day of L-triiodothyronine (molar ratio of triiodothyronine: thyroxine = 1:1), and one with 0.021 mg/kg/day of L-triiodothyronine (molar ratio of triiodothyronine: thyroxine = 1:3.5).

Results. The results of the antigoitrogenic test are shown graphically in Fig. 1. As judged by the relative thyroid weights, ex-

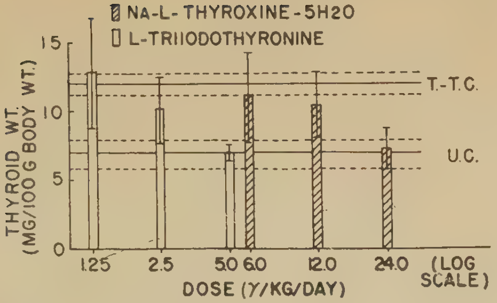


FIG. 1. Thyroid gland weights (mean ± S.D.) of thiouracil-treated rats given subcut. injections of 3 dose levels of either sodium-L-thyroxine pentahydrate or L-triiodothyronine. The mean (solid line) and stand. dev. (broken line) of the thyroid gland weights of both the untreated control (U.C.) and thiouracil-treated control (T.-T.C.) rats is given for reference purposes.

pressed as mg/100 g body weight, both the 1.25 γ of L-triiodothyronine and the 6 γ of L-thyroxine sodium pentahydrate failed to produce a significant change in weight of the thiouracil-induced goitre. On the contrary, the rats treated with the 5 γ dose of triiodothyronine, or the 24 γ dose of thyroxine sodium pentahydrate, had thyroid glands equivalent in weight to those of normal, untreated control rats. The goitrogenic effect of the thiouracil was, therefore, completely inhibited by these doses.

In Fig. 2 is shown the comparative mean change in oxygen consumption induced by the various respective doses of L-thyroxine sodium pentahydrate and L-triiodothyronine, when given chronically by daily injection. The values as plotted represent the mean change in oxygen consumption of the treated rats, using the mean oxygen consumption of thyroidectomized control rats as the baseline. The mean difference in oxygen consumption between the intact control rats and thyroidectomized control rats was 1.15 l/sqm/hr. Neither 6 γ of thyroxine sodium pentahydrate nor 1.25 γ of triiodothyronine elevated the oxygen consumption of the treated thyroidectomized rats to the level of the intact controls.

The rats with 2.5 γ of triiodothyronine reached the intact control level after 14 days of treatment, but those treated with 12 γ of thyroxine sodium pentahydrate attained this same level only by the seventeenth day. In the next higher dosage level, it was again evident that the animals treated with 5 γ of triiodothyronine reached the oxygen consumption level of the intact control rats earlier (10 days) than the ones treated with 24 γ of thyroxine sodium pentahydrate (14 days). The highest doses of both agents (12.5 γ of triiodothyronine and 60 γ of L-thyroxine sodium pentahydrate) quickly increased the oxygen consumption into the hyperthyroid range. Furthermore, with this 1:3.5 dosage ratio of triiodothyronine to thyroxine, the triiodothyronine produced a definitely greater response ($P = <0.05$ by the “t” test(5) for the 10th, 14th, and 17th days) than the thyroxine. These data indicate, therefore, that within the hypothyroid and euthyroid ranges, the molar

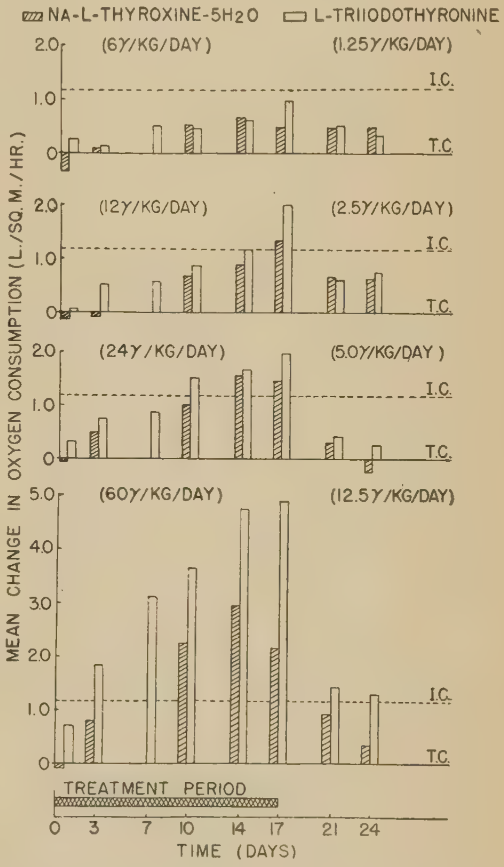


FIG. 2. Mean change in oxygen consumption induced by 4 dose levels of either sodium-L-thyroxine pentahydrate or L-triiodothyronine, when administered by daily inj. The mean oxygen consumption of each thyroidectomized control (T.C.) and intact control (I.C.) rats is given for reference purposes.

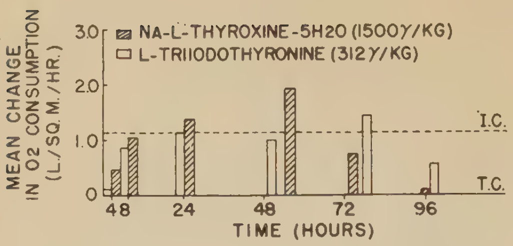


FIG. 3. Mean change in oxygen consumption induced by either sodium-L-thyroxine pentahydrate or L-triiodothyronine when administered by a single inj. at zero time.

potency ratio of L-triiodothyronine to L-thyroxine is close to 3.5:1, but that for the hyperthyroid range the ratio may be greater.

The calorigenic effect produced by a single injection of either agent is shown in Fig. 3. The response to 1500 γ/kg of L-thyroxine sodium pentahydrate and to 312 γ/kg of L-triiodothyronine was of a similar magnitude. The thyroxine gave a greater response 52-56 hours after the injection ($P = <0.05$ by the "t" test), but this relationship was reversed after about 75 hours.

Table I summarizes the results obtained when these two agents were injected into intact rats. The rats which received the triiodothyronine in a 1:1 molar dosage ratio of thyroxine sodium pentahydrate to triiodothyronine (Group 3) showed a mean oxygen consumption of 9.1 l/sqm/hr. This is not much different from the 8.4 l/sqm/hr ($P = <0.3$; >0.2) of the thyroxine-treated animals (Group 2). Furthermore, the mean oxygen consumption (8.0 l/sqm/hr) of rats treated with triiodothyronine at the 3.5:1 molar ratio of thyroxine sodium pentahydrate to triiodothyronine (Group 4), was only slightly less than that of the thyroxine-treated rats.

Discussion. The antigoitrogenic test indicates that, on a molar basis, triiodothyronine is 3½ times as potent as thyroxine. These results are in agreement with the original publication of Gross and Pitt-Rivers(1). Observations on the effect of these agents on oxygen consumption indicate that the respective calorigenic responses induced by these agents are again 3.5:1 when they are not above the euthyroid range, and therefore are in agreement with the results of the antigoitrogenic test. However, when the oxygen consumption rises to the hyperthyroid range, the activity ratio is greater than 3.5:1. Intact rats apparently react differently (quantitatively) to triiodothyronine than to thyroxine for the 1:1 molar dosage ratio of thyroxine to triiodothyronine gave results not too far different from the 3.5:1 ratio. The results obtained with the 1:1 dosage ratio confirm in part, the work reported by Gemmill(3) in which he stated that there was little difference in the response induced by the two agents in intact rats. However, since in the intact animal, neither dose of triiodothyronine, gave results too dissimilar from those produced by thyroxine, one must consider possible conversion of triiodothyronine to a less active derivative by the thyroid gland. In such a case measurements in the intact animal would not be a true indication of the inherent calorigenic potency of triiodothyronine itself. This latter possibility is in agreement with the suggestion of Roche(6) that triiodothyronine and thyroxine may both be synthesized by the thyroid gland. It is also in

TABLE I. Effect of L-Thyroxine Sodium Pentahydrate and 3:5:3′-L-Triiodothyronine on Oxygen Consumption of Intact Rats.

Group No.	Treatment	Molar dosage ratio*	No. of rats	Oxygen consumption† ($l/m^2/hr$)
1	Control		6	6.5 ± 0.4
2	L-thyroxine sodium pentahydrate (0.1 mg/kg/day)		6	8.4 ± 0.9
3	3:5:3′-L-triiodothyronine (0.073 mg/kg/day)	1:1	6	9.1 ± 1.0
4	3:5:3′-L-triiodothyronine (0.021 mg/kg/day)	3.5:1	6	8.0 ± 0.3

* Thyroxine : triiodothyronine.
† Mean ± S.D.

accord with the work of Keating and Albert (7) who state that following radioactive triiodothyronine treatment, there is a greater accumulation of I^{131} in the thyroid than after radioactive thyroxine treatment.

Summary. Antigoitrogenic tests in rats showed L-triiodothyronine to be about 3.5 times as active as L-thyroxine on a molar basis. In addition, in thyroidectomized rats, the L-triiodothyronine had a more potent calorigenic effect than L-thyroxine; in this case, the molar potency ratio was not less than 3.5:1. When the response was in the hyperthyroid range, the ratio was even greater than 3.5:1. Calorigenic tests in normal, intact rats showed a somewhat different effect in that both the triiodothyronine at a 1:1 molar dosage ratio and the triiodothyronine at 1:3.5 molar dosage ratio (triiodothyronine:thyroxine) produced changes not too far different from that obtained with the thyroxine.

The 3:5:3′-L-Triiodothyronine used in this study was prepared by Glaxo Laboratories Ltd., England.

We thank Miss Mary F. Sax for performing the surgical thyroidectomies, and Mr. Alfred Boris for his valuable technical assistance throughout the entire course of this study.

1. Gross, J., and Pitt-Rivers, R., *Lancet,* 1952, v1, 593.

2. Gross, J., Pitt-Rivers, R., and Trotter, W. R., *Lancet,* 1952, v1, 1044.

3. Gemmill, C. L., *Am. J. Physiol.,* 1953, v172, 286.

4. Hill, A. V., and Hill, A. M., *J. Physiol.,* 1913, v46, 81.

5. Freeman, H. A., *Industrial Statistics,* John Wiley and Sons, New York, 1942, 5th Printing, 1949, p48.

6. Roche, J., and Lissitzky, S., and Michel, R., *Compt. rend. soc. biol.,* 1952, v146, 1474.

7. Keating, F. R., and Albert, A., *J. Clin. Invest.,* 1953, v32, 580.

Received July 30, 1953. P.S.E.B.M., 1953, v83.

Dextran, an Antigen in Man.* (20521)

Paul H. Maurer. (Introduced by F. J. Dixon.)

From the Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh.

Kabat and Berg have recently reported(1)† that various dextran preparations are antigenic in man. Quantitative immunochemical studies were presented on the reaction of various native and partially hydrolized dextrans with homologous human antidextran sera. As the implications of these original findings to the use of dextran as a blood substitute are manifold, it was agreed to have the results checked independently in another laboratory. Dextrans have been shown to cross react with types II, XII, and XX horse and rabbit antipneumococcal sera(2-4). Therefore, it is conceivable that the individuals who had high concentrations of precipitins and showed skin sensitivity to dextran may have produced antibodies to a cross reacting pneumococcal polysaccharide. A complete analysis for antibodies to these polysaccharides was performed in both the pre- and post-immunization sera.

The persistence of antibodies to many pneumococcal polysaccharides has been shown by Heidelberger, *et al.*(5-8). However, the pneumococcal polysaccharides(9), in contrast to the dextrans(10,11), are not metabolizible and may persist for some time. It was of great interest, therefore, to see what the concentration of antibodies against a metabolizible polysaccharide would be a year after the antigen was injected.

Experimental dextrans. Five samples of native dextran were used. Three of these, B 1254, B 742, and B 512, were obtained from the Northern Regional Research Laboratory, U. S. Department of Agriculture, Peoria, Ill.

---

[*] This investigation was supported by a grant from the Office of the Surgeon General, Department of the Army contract No. DA-49-007-MD-248 upon recommendation of the Subcommittee on Shock of the National Research Council.

[†] The author wishes to thank Dr. E. A. Kabat for making his manuscript available before publication.

This is Reprint No. 26 of the Pathology Department, University of Pittsburgh School of Medicine.

TABLE I. Some Chemical and Physical Properties of Dextran Samples.

Dextran sample	Source	N, %	Branching ratio, $\frac{1:6}{\text{non } 1:6}$ linkages	Molecular wt
		Native		
B 1254	NRRL*	<.01	11	
B 742	NRRL	<.01	1.9	
B 512	NRRL	.01	21	
3079 (Swedish)	NRC†	.05	7.1	95000000
N 279	CSC‡	.018	18	1940000
		Clinical		
B 742	NRRL	<.01	1.9	
OP 163 (Swedish)	NBS§		7.1	28300
R 229	CSC	.005	32-49	53000
258 A 7‖	CSC	.009	32-49	83000

Analytical data obtained from source:

* Northern Regional Research Laboratory.

† National Research Council.

‡ Commercial Solvents Corp.

§ Nat. Bureau of Standards.

‖ Not a routine clinical dextran preparation. Prepared for special test purposes for the NRC.

through the courtesy of Mr. C. E. Rist and Dr. A. Jeanes. These are dextrans with different degrees of branching, the B 1254 from *Streptococcus dextranicum* and the other two from strains of *Leuconostoc mesenteroides*. Dextran sample number 3079 was a native preparation obtained from Pharmacia Co. through the National Research Council. Sample 279 was a native dextran supplied by Drs. F. Schulz and M. E. Bachman of Commercial Solvents Corp. This was prepared from the NRRL B 512 strain and similar to sample B 512. Four samples of clinical dextran were available. Swedish sample OP 163 was obtained from Dr. E. A. Kabat. Samples R 229 and 258 A 7 were supplied by Commercial Solvents Corp. B 742 was obtained from the Northern Regional Research Laboratory. A summary of chemical and physical properties of some of the dextrans is given in Table 1 of reference 1. The properties of dextrans used in this study are given in Table I. Samples of C polysaccharide (S 146) from a type I pneumococcus, and the type specific pneumococcal polysaccharides from types II (Squibb) and type XII (Squibb 115 A) were kindly supplied by Dr. M. Heidelberger. Type XX pneumococcal polysaccharide was obtained through the courtesy of Dr. Rachel Brown of the New York State Department of Health.

Analytical solutions of dextran were prepared by dissolving samples dried to constant weight over P_2O_5 *in vacuo*. Solutions were kept with a drop of chloroform. For injections, suitable dilutions were made in 0.85% saline containing 0.25% phenol and tested for sterility.

Immunization procedure. Healthy medical student volunteers were used. The procedures followed for pre-immunization bleedings, intracutaneous skin testing, injections, post-immunization bleedings, and skin testing were those outlined in reference 1. All serum samples were handled with sterile precautions and in addition 'Merthiolate' (0.01%) and phenol (0.25%) were added.

Quantitative precipitin studies. The pre- and post-immunization serum samples were assayed for antibody nitrogen by the quantitative precipitin method of Heidelberger and MacPherson(12). A complete description of the procedure is in reference 1. Results are given in μg antibody nitrogen precipitated per ml of serum although 3 ml samples were used for analysis (Table II). Quantitative precipitin curves were obtained with sera obtained from individuals who had shown the best antibody response. Their sera, both from pre- and post-immunization bleedings were analyzed for antibody N against C polysaccharide from type I pneumococcal poly-

TABLE II. Response of Humans to Injection of Dextran (1 mg).

		Nitrogen precipitable by dextran from serum		Cutaneous reaction	
Dextran sample used	Subject	Pre-immunization, µg N/ml	Post-immunization, µg N/ml	Pre-immunization	Post-immunization
CSC clin. R 229	1	—	.5	—	—
	2	—	0	—	—
	3	—	.1	—	—
	4	—	0	—	—
	5	—	0	—	—
	6	—	.1	—	—
	7	—	.5	—	—
	8	—	1.6	—	—
	9	—	.1	—	—
	10	—	.7	—	—
	11	—	0	—	—
	12	—	.1	—	—
	13	—	0	—	—
CSC clin. 258 A 7	14	.3	10.7	—	++++
	15	.1	12.1	—	++++
	16	.4	9.6	—	+++
	17	.6	.4	—	—
	18	.0	.3	—	—
	19	.1	.8	—	—
CSC native N 279	20	1.2	3.5	—	—
	21	1.8	7.1	—	+++
	22	0	0	—	—
	23	0	.3	±	—
	24	0	2.2	—	—
Swedish clin. 163	25	.4	9.9	—	++
	26	.2	24.0	—	++++
	27	0	.8	—	—
	28	.1	1.1	—	—
	29	.2	6.6	±	+
	30	.2	3.1	—	—
Swedish 3079	31	0	1.4	—	+
	32	0	1.5	±	+++
	33	0	0	+	—
	34	1.5	17.3	+	++++
	35	1.9	2.4	—	—
	36	1.6	13.1	+±	++++
NRRL B 742 (clin.)	37	0	0	—	—
	38	.3	4.8	—	—
	39	.2	.9	—	—
	40	.4	.7	—	—
	41	.2	.3	—	—
NRRL B 742 (native)	42	.4	.4	—	—
	43	3.0	3.9	+++	++
	44	5.1	8.3	+++	++++
	45	2.2	2.8	+	++
NRRL B 1254 (native)	46	1.4	.8	—	++
	47	.5	.9	+	+++
	48	0	.6	—	—
	49	.5	4.4	+++	++++
	50	.8	.9	—	++
NRRL B 512 F (native)	51	3.1	21.8	+++	++++
	52	.5	3.7	—	+++
	53	1.6	9.2	+	++++
	54	.3	.7	+	+
	55	.6	1.1	—	—

saccharide and pneumococcal polysaccharides, types II, XII, and XX. The methods for analysis were described by Heidelberger *et al.*(5).

Results. Table II summarizes the results obtained in 55 individuals immunized with the various dextrans. They agree well with those reported by Kabat and Berg. A substantial number of volunteers showed rises in nitrogen precipitable by dextran in the post-immunization serum as compared with that in the pre-immunization sample. There was no significant reaction in any of the 13 volunteers to the CSC clinical dextran R 229. Although this would seem to indicate that this preparation is non-antigenic, it is quite possible that these individuals are not "reactors." Kabat and Berg encountered a similar observation and found their group of nonreactors to CSC clinical dextran to be nonreactors to a second course of CSC clinical dextran and also to CSC native dextran. The reaction noted in 3 of 6 volunteers given the CSC clinical dextran 258 A 7 does not mean that CSC clinical dextran is highly antigenic. This preparation was not a regular clinical preparation but was prepared for special test purposes for the National Research Council. As can be seen from Table I, it has a higher average molecular weight than that of the CSC R 229. Three out of 5 students injected with CSC native 279 showed a significant increase in antibody N in the post-immunization sera. As discussed by Kabat and Berg, an increase of 2 μg or more in precipitable nitrogen per ml from the pre- to post-immunization sample is considered significant. From the rest of Table II it is evident that there are good reactors to all the remaining dextran preparations. It is also of interest that 3 of the 4 individuals injected with the most highly branched native dextran, B 742, had relatively high levels of precipitins in their serum before immunization and not much higher levels after immunization. The presence of significant precipitin levels to NRRL B 742 native dextran in most human sera (about 3-5 μg antibody N per ml) has been confirmed in about 250 sera received from Army volunteers(13).

Comparison of the post-immunization precipitin levels with the skin tests showed that 19 out of 21 individuals with 2 μg antibody N per ml or more had positive skin tests and of the 36 individuals, with antibody levels less than 2 μg N per ml, 31 were negative or ±. This difference is highly significant as pointed out in(1). Furthermore, it is evident from Table II that a definite antibody response on immunization was accompanied by the appearance of cutaneous erythema and wheal reactions in individuals who had negative or ± skin tests before immunization. In those cases where initial sensitivity existed, an increase in antibody level was usually accompanied by an increased skin reaction.

As discussed by Kabat and Berg, the number of individuals injected with each dextran is not sufficient to permit inferences to be drawn as to the relative antigenicity of each dextran sample.

Results obtained on the complete quantitative precipitin curves from the best anti-dextran sera will not be reported. Suffice to say that a summary of the observations reported here are similar to those reported in (1). 1—Native dextrans precipitated more antibody that the clinical dextran fractions. 2—Inhibition of precipitation by excess antigen required smaller quantities of the clinical dextran than of the native dextrans. 3—Among the native dextrans, B 742, which was the most highly branched, required much larger quantities to precipitate equivalent amounts of antibody nitrogen than used for other native dextrans.

The values presented in the last 2 columns in Table III indicate that there is not much difference in the anti-dextran antibody nitrogen level before or after absorption with the various cross reacting pneumococcal polysaccharides. It is also significant to note that immunization with the dextran did not cause much of a change in the antibody levels to the specific pneumococcal polysaccharides. Table IV presents the data showing that significant amounts of antibodies to the various dextrans in some of the sera persist one year after immunization.

Discussion. The data presented clearly confirm the original findings of Kabat and Berg that the injection of small amounts (1 mg) of native or clinical dextran into hu-

TABLE III. Micrograms Antibody N Precipitated per ml of Serum from Medical Volunteers Receiving Injections of Various Dextrans.

Subject No.	Inj. with dextran	Bleeding	Anti-				Anti-dextran after absorption with pneumococcal polysaccharides	Anti-dextran before absorption with pneumococcal polysaccharides
			CI	SII	SXII	SXX		
15	OSC clin. 258 A 7	Pre-immunization	2.0	1.4	2.6	.0	.4	.1
		Post "	2.2	2.9	1.3	.1	10.7	11.5
21	CSC native N 279	Pre "	.3	1.1	.9	1.0	.2	1.8
		Post "	1.2	1.4	.7	1.0	5.9	6.0
26	Swedish clin. OP 163	Pre "	1.6	1.4	.8	.0	.5	.2
		Post "	1.0	.4	.4	.0	19.8	19.8
34	Swedish native 3079	Pre "	.9	.5	.9	.5	1.1	1.5
		Post "	1.0	1.3	.4	.6	14.9	16.2
38	NRRL B 742 clin.	Pre "	1.3	1.7	4.0	1.7	1.8	.3
		Post "	1.2	3.0	3.7	3.5	3.8	4.4
44	NRRL B 742 native	Pre "	1.5	.0	.6	.6	3.0	5.1
		Post "	1.3	.0	1.6	1.2	6.5	6.3
49	NRRL B 1254	Pre "	.7	2.8	.0	.0	.8	.5
		Post "	.5	2.9	.4	.7	4.1	4.1
51	NRRL B 512 F	Pre "	1.6	.0	1.2	.3	2.7	3.1
		Post "	1.2	.0	.0	.5	19.4	17.1

mans leads to the production of specific antibodies. It would appear then that the allergic reactions reported previously(14-16) are due to the antibodies to dextran. However, it should be borne in mind that the response of an individual or animal to a small dose of antigen is not necessarily the same as that elicited upon exposure to a massive infusion or infusions. The phenomenon of "immunological paralysis" which has been reported by other workers with polysaccharide antigens (17,18) cannot be overlooked. Experiments are now underway in our laboratory which would indicate that it is possible to fatigue the immune response in rabbits to bovine serum albumin and human serum albumin (19) by repeated massive injections of the protein solution. It is realized that inferences should not be drawn from studies on experimental animals to the human.

The possible explanation advanced by Kabat and Berg that individuals who showed precipitins and skin sensitivity to dextran may conceivably have produced antibodies to some cross reacting substance such as type II, XII, or XX pneumococcal polysaccharides has to be ruled out at least as far as the pneumococcal polysaccharides are concerned. This would be more evidence in favor of the conclusions advanced that the antibodies produced after injection of dextran are actually specific antibodies against the dextrans.

The persistence of antibodies to dextran (Table IV) parallels closely the persistence of antibodies to the pneumococcal polysaccharides. It has been shown and discussed (20) that "contrary to the behavior of antitoxin in the human subject which drops off to one-half or less during the second week period and continues to decrease at a slower rate, the type specific anti-carbohydrate in human sera remains level at or near the top for 5-8 months."

Explanations advanced for the difference in behavior are that toxins and toxoids being proteins are attacked by tissue enzymes whereas the pneumococcal polysaccharides are extremely resistant to attack. The persistence of antigen (polysaccharide) explains the production of antibody according to the Breinl-Haurowitz-Mudd-Alexander-Pauling group. According to Burnet, the presence of antigen is necessary only for the initial modification of the antibody producing mechanism. Antigen need not persist for antibody production to occur. A compromise between the 2 theories has been advanced by Heidelberger to explain both the anamnestic reaction observed with toxoid and not with polysaccharide antigen, and the persistence of antibody levels to

TABLE IV. Persistence of Antibodies in Human Sera after Injection of Various Dextrans.

Subject	Inj. with dextran	Date of bleeding	μg antibody nitrogen per ml serum
15	OSC clinical 258 A 7	5/28/52	12.1
		6/26/52	11.5
		2/ 9/53	9.0
21	CSC native	2/ 5/52	7.1
		2/25/52	6.0
		3/ 5/53	6.0
26	Swedish clinical	2/25/52	24.0
		3/18/52	19.8
		3/ 5/53	12.3
34	Swedish native	2/ 6/52	17.3
		2/25/52	16.2
		3/ 5/53	15.6
38	NRRL B 742 clinical	2/25/52	4.8
		3/19/52	4.4
		3/ 5/53	4.2
44	NRRL B 742 native	2/14/52	8.3
		3/20/52	6.3
		3/ 5/53	5.8
51	NRRL B 512 F	2/25/52	21.8
		3/19/52	17.1
		3/ 5/53	18.0

the polysaccharide antigen(20).

The persistence of antibodies to dextran after a year or so does not aid in the elucidation of this problem. It would appear that here, a metabolizible polysaccharide antigen injected in so small an amount (1 mg) should certainly be gone in the course of a few weeks and therefore lend support to the Burnet theory. However, Neill, Hehre, and coworkers(2) have shown that dextran is a contaminant of sugar samples and also that the human gastrointestinal tract contains dextran-producing organisms(21). In addition, several investigators have reported the presence of dextran in various tissues(22,23). It is quite possible that these factors may be providing a sufficient antigenic stimulus to keep the antibody level up.

Summary. It has been confirmed that native and clinical dextrans are antigenic in man. Three weeks after injection of 1 mg of dextran precipitins and cutaneous wheal and erythema sensitivity developed. The presence in human sera of antibodies to the pneumococcal polysaccharides which cross react with dextrans in other species (rabbit and horse) appears to have little to do with the reaction of humans to dextran. Antibodies produced after immunization with dextran have been shown to be specific for dextran. The persistence of antibodies to dextran has been shown.

The author wishes to express his thanks to Drs. E. A. Kabat, Gerard Turino, and F. Douglas Lawrason of the Subcommittee on Shock of the National Research Council for advice and help in obtaining many of the materials used in this study.

---

1. Kabat, E. A., and Berg, D., *J. Immunol.*, 1953, v70, 514.

2. Neill, J. M., Hehre, E. J., Sugg, J. Y., and Jaffe, E. M., *J. Exp. Med.*, 1939, v70, 429.

3. Sugg, J. Y., and Hehre, E. J., *J. Immunol.*, 1942, v43, 119.

4. Neill, J. M., Sugg, J. Y., Hehre, E. J., and Jaffe, E., PROC. SOC. EXP. BIOL. AND MED., 1941, v47, 339.

5. Heidelberger, M., MacLeod, C. M., Kaiser, S. J., and Robinson, B., *J. Exp. Med.*, 1946, v83, 303.

6. Heidelberger, M., and DiLapi, M. M., *J. Immunol.*, 1949, v61, 153.

7. Heidelberger, M., DiLapi, M. M., Siegal, M., and Walter, A. W., *J. Immunol.*, 1950, v65, 535.

8. Heidelberger, M., MacLeod, C. M., DiLapi, M. M., *J. Immunol.*, 1951, v66, 145.

9. Heidelberger, M., Kendall, F. E., and Scherp, H. W., *J. Exp. Med.*, 1936, v64, 559.

10. Bloom, W. L., and Wilcox, M. L., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 3.

11. Hellman, L., and Becker, D., 1952, Reports to National Research Council Subcommittee on Shock.

12. Heidelberger, M. and MacPherson, C. F. C., *Science*, 1943, v97, 405; v98, 63.

13. Maurer, P. H., Unpublished experiments.

14. Bohmansson, G., Rosenquist, H., Thorsen, G., and Wilander, D., *Acta Chir. Scand.*, 1946, v94, 149.

15. Turner, F. P., Butler, B. C., Smith, M. G., and Scudder, J., *Surg., Gyn., and Obst.*, 1949, v88, 661.

16. Pulaski, E. J., *et al.*, 1951, Reports of National Research Council Subcommittee on Shock.

17. Felton, L. D., and Ottinger, B., *J. Bact.*, 1942, v43, 94.

18. ———, *J. Immunol.*, 1949, v61, 107.

19. Dixon, F. J., and Maurer, P. H., *Fed. Proc.*, 1953, v12, 441.

20. Heidelberger, M., in *The Nature and Significance of the Antibody Response*, Chap. 5, Columbia University Press, New York, 1953.

21. Hehre, E. J., and Neill, J. M., *J. Exp. Med.*, 1946, v83, 147.

22. Terry, R., and Yulie, C. L., *Fed. Proc.*, 1952, v11, 430.

23. Reports to National Research Council Subcommittee on Shock, 1952-1953.

Received July 30, 1953. P.S.E.B.M., 1953, v83.

AUTHORS' INDEX

VOLUME 83

(*The numerals indicate the page*)

Adams, J. M. 308.
Aggeler, P. M. 69, 384.
Aldrich, R. A. 566.
Allison, J. B. 398, 438.
Almquist, H. J. and Merritt, J. B. Effect of crystalline trypsin on raw soybean growth inhibitor. 269.
Anderson, E. C. and Magee, D. F. Effect of feeding fast on duration of thiopental anesthesia. 110.
Andrews, G. A. 376.
Atkinson, W. B. 405.
Awapara, J. 367.
Axelrod, A. E. and Martin, C. J. Biochemical changes in thermally-injured cutaneous tissue. Susceptibility to proteolytic enzymes and extractability of collagen. 463; 269, 461.

Bachtold, J. G. 807.
Baeder, D. H. 468.
Bahner, C. T. 801.
Baker, F. H., Mac Vicar, R., Pope, L. S. and Whitehair, C. K. Placental and mammary transfer of vitamin A and carotene by beef cows. 571.
Baker, R., Reaven, G. and Sawyer, J. Effect of toluidine blue on metastatic calcification. 281.
Balch, H. E. 683.
Ballin, J. C. 6, 10.
Banerjee, S., Belavady, B. and Mukherjee, A. K. Effect of dehydroascorbic acid in rabbits. 133.
Barker, S. B. and Schwartz, H. S. Further studies on metabolism of tissues from thyroxine-injected rats. 500.
Barnafi, L. 784.
Baron, L. S., Formal, S. B. and Spilman, W. Use of Vi phage lysates in genetic transfer. 292.
Bartholomew, R. J. A simplified method for preparation of corticotropin. 334.
Bartlett, R. G. and Register, U. D. Effect of cold and restraint on blood and liver nonprotein sulfhydryl compounds. 708.
Bartlett, R. G., Jr., Helmendach, R. H. and Bohr, V. C. Effect of emotional stress, anesthesia, and death on body temperature of mice exposed to cold. 4.
Bass, D. E. 660.
Batchelor, E. 43.
Bateman, G. Q. 458.
Baume, L. J. and Frandsen, A. S. Phase contrast microscope study of oral epithelium of normal and vitamin A-deficient rats. 356.
Beard, D. 479.
Beard, J. W. 113, 479.
Beaton, G. H., Ozawa, G., Beaton, J. R. and McHenry, E. W. Effect of anterior pituitary growth hormone on certain liver enzymes. 781.
Beaton, J. R. 781.
Beck, E. M. and Ralli, E. P. Influence of dietary factors and the adrenal on hyaluronidase spreading reaction in the rat. 588.
Beckfield, W. J. 468.
Beecher, L. 603.
Belavady, B. 39, 133.
Bennett, A. 847.
Beraldo, W. T. 455.
Berg, E. Effect of castration in male mice on *Schistosoma mansoni.* 83.
Berg, O. and Gorbman, A. Utilization of iodine by the thyroid of the platyfish, *Xiphophorus (Platypoecilus) maculatus.* 751.
Bergstrom, S. and Norman, A. Metabolic products of cholesterol in bile and feces of the rat. Steroids and bile acids. 71.
Berkowitz, R. D. 311.
Bernfeld, P., Donahue, V. M. and Homburger, F. Characteristic individual electrophoretic patterns in humans. 429.
Bernfeld, P., Stefanini, M., Berkowitz, R. D. and Hennessey, F. B. Electrophoretic plasma protein patterns in families with hemophilia. 311.
Biddulph, C. 458.
Binns, W. 458.
Bishop, C. and Beecher, L. Effect of phenylbutazone on uric acid excretion in patients with gout and rheumatoid arthritis. 603.
Blackford, M. E. 520.
Bo, W. J. and Atkinson, W. B. Histochemical studies on glycogen deposition in uterus of the rat. Effect of starvation. 405.
Bock, F. 338.
Bodine, J. H. and Carlson, L. D. Sulfhydryl and disulfide. Reactive sulfhydryl and disulfide groups of protyrosinase and tyrosinase. 717.
Bohr, V. C. 4.
Boldt, R. E. 135.
Bondi, A., Kornblum, J. and Phalle, M. Des. Isolation of penicillin-susceptible mutants from penicillinase-producing strains of *M. pyogenes.* 527.
Bondurant, J. 871.
Bovarnick, M., Fieber, S., Bovarnick, M. R. and Kazlowski, J. Rate of excretion of glutamyl polypeptide and its polymers in humans. 253.
Bovarnick, M. R. 253.
Bowman, D. E. Proteolytic, hypotensive and thromboplastic properties of hypochlorite treated trypsin. 242.
Brandenburger, E. A. 221.
Braude, A. I., Davey, P. and Siemienski, J. Prevention of blood coagulation by antibiotics *in vitro.* 160
Brecher, G. A. and Praglin, J. A modified bristle flowmeter for measuring phasic blood flow. 155.
Breese, S. S., Jr. and Briefs, A. Certain physical properties of a herpangina strain and a pleurodynia strain of Coxsackie virus. 119.
Briefs, A. 119.
Brockman, R. W. 1.
Brodie, E. C. 254.
Brown, G. C., Craig, D. E. and Kandel, A. Effect of benzimidazole on experimental poliomyelitis in mice and monkeys. 408.
Brown, J. H. U. Influence of the drug DDD on adrenal cortical function in adult rat. 59.

Brown, L. V. 170.
Brueckner, A. L. 793.
Buchsbaum, R. 30.
Buckaloo, G. W. 618.
Burchenal, J. H. 369.
Burr, M. M. and Nagler, F. P. Mumps infectivity studies in hamsters. 714.
Burton, S. 212.
Buso, R. 681.
Bussell, R. H. 671.
Butler, L. C. and Morgan, A. F. Eosinopenic responses of pyridoxine-deficient rats to epinephrine, adrenocorticotropic hormone and hypoxia. 655.
Butts, D. C. A. Ch hemagglutinogen in primates. 701.

Campbell, E. W. and Stefanini, M. Isolation and purification of plasma thromboplastin: Its physico-chemical and biologic properties. 105.
Cantarow, A. 181.
Carlson, L. D. 717.
Carmichael, N. 676.
Carpenter, F. G. Suppression of hyperoxic convulsions by nitrogen at high pressure. 546.
Casals, J. and Brown, L. V. Hemagglutination with certain arthropod-borne viruses. 170.
Case, J. D., Aldrich, R. A. and Neve, R. A. Sedormid-induced porphyria in the rat. 566.
Cassidy, J. W. 674.
Castaneda, M. R. Surface fixation as a practical method for diagnosis of Brucellosis in man and animals. 36.
Chaikoff, I. L. 498, 756.
Chassin, J. L., McDougall, H. A., MacKay, M. and Localio, S. A. Effect of stress upon the healing of wounds in rats. 798.
Cheever, F. S. and Dickos, J. Propagation of St. Louis encephalitis virus in cells of the Ehrlich ascitic tumor of mice. 822.
Chitale, R. P. 39.
Christensen, H. N. and Riggs, T. R. Amino acids and peptides in the oviduct of the laying hen. 697.
Clausen, H. J. Experimental production of struma lymphomatosa. 835.
Cleverdon, R. C. 599.
Cohen, A. M. and Rachmilewitz, M. Effect of aureomycin in rats with chronic alloxan diabetes. 50.
Cohn, S. H. and Gong, J. K. Effect of chemical agents on skeletal content and excretion of injected Strontium^{89}. 550.
Cole, B. A. and Kent, J. F. Immobilization of *Endamoeba histolytica in vitro* by antiserum produced in the rabbit. 811.
Coleman, V. R., Gunnison, J. B. and Jawetz, E. Participation of erythromycin and carbomycin in combined antibiotic action *in vitro.* 668.
Combs, G. F. 17.
Cooperman, J. M. 88.
Corcoran, A. C. 631.
Costello, M. J. 266.
Coulter, E. P. 266.
Coutinho, H. B. 809.
Craig, D. E. 408.
Crossley, M. L., Allison, J. B., Parker, R. P., Kuh, E. and Seeger, D. R. Chemotherapy of tumors in rats with certain ethylenephosphoramides. 438.
Crossley, M. L., Allison, J. B., Wannemacher, R., Migliarese, J., Parker, R. P., Kuh, E., Seeger, D. R. and Partridge, R. Distribution of P^{32} following injection of TEPA in rats. 398.
Croxatto, H., Vera, C. and Barnafi, L. Inactivation of antidiuretic hormone by blood serum of the pregnant woman. 784.
Culbertson, C. G. 161.

D'Angelo, S. A., Stevens, C. E., Paschkis, K. E. and Cantarow, A. Influence of cortisone on thyroid-pituitary interaction in normal and goitrous rats. 181.
Daniel, G. E. and Park, H. D. Reproduction in paramecium as affected by small doses of X-ray and beta radiation. 662.
Daniel, L. J. and Gray, L. F. Molybdenum toxicity in *Lactobacillus leichmannii.* 487; 625.
Dauben, W. G. 498.
Davey, P. 160.
Dawson, A. B. 509.
Deichmiller, M. P. 287.
Deiss, W. P. 506.
Del Greco, F. 631.
Denton, C. A. 221.
Deringer, M. K., Dunn, T. B. and Heston, W. E. Results of exposure of strain C_3H mice to chloroform. 474.
Des Phalle, M. 527.
Devitt, J. E., Pirozynski, W. J. and Samuels, P. B. Mast cell resistance to hormonal influence. 335.
Dewey, V. C. and Kidder, G. W. Further studies on effect of folic acid analogs on growth of tetrahymena. 586.
Dickos, J. 822.
Dienes, L. L type cultures isolated from streptococci. 579.
Dixon, F. J., Maurer, P. H. and Deichmiller, M. P. Half-lives of homologous serum albumins in several species. 287; 163.
Donahue, V. M. 429.
Donaldson, D. M. 184.
Done, A. K., Ely, R. S., Heiselt, L. R. and Kelley, V. C. Circulating 17-hydroxycorticosteroids in ascorbic acid-deficient guinea pigs. 722.
Dorfman, R. I. 223.
Dounce, A. L. and Kay, E. R. M. Attempted enzymatic phosphorylation of ribonucleic acid. 321.
Drallmeier, J. L. 520.
Drucker, R. E. 88.
Du Buy, H. G. 62.
Duncan, G. D., Gray, L. F. and Daniel, L. J. Effect of zinc on cytochrome oxidase activity. 625.
Dunn, T. B. 474.

Eadie, G. S. 111.
Eckert, E. A., Reeve, R. H. and Beard, J. W. Measurement of complement fixation in small volumes. 113.
Eddie, B. 99.
Ehrich, W. E. 468.
Eisenhauer, E. R. 214.

Eisenstein, A. B. Steroid compounds resulting from incubation of cortisone with surviving liver slices. 27.
Elvehjem, C. A. 135.
Ely, R. S. 722.
Emery, B. E. 69.
Engel, F. L. and Hewson, K. Fatty acid and ketone metabolism during hemorrhage and shock in the rat. 608; 568.
Epstein, E. and Quick, A. J. Effect of injecting platelet extract intravenously. 453.
Erslev, A., Lavietes, P. H. and Van Wagenen, G. Erythropoietic stimulation induced by "anemic" serum. 548.
Etheridge, J. 871.
Evans, H. J. Action of cortisone on developing chick embryo. 31.
Everett, J. W. 820.
Everett, M. R. 75.

Fang, H. S. 98.
Farrell, G. L. and Richards, J. B. Isolation of a potent sodium-retaining substance from adrenal venous blood of the dog. 628.
Favour, C. B., Murray, J. E., Wemyss, C. T., Jr. and Miller, B. F. Serum complement levels in dogs undergoing kidney homotransplantation. 352.
Feinstein, R. N. and Ballin, J. C. Effect of whole body x-irradiation on a natural inhibitor of carboxypeptidase. 6; and Ballin, J. C. Carboxypeptidase in mammalian tissue. 10.
Feldman, D. 858.
Fieber, S. 253.
Figge, P. H. J. 264.
Figueroa, E. S. and Seibert, F. B. Chromatography of *α-ε*-diaminopimelic acid on starch columns. 535.
Fillerup, D. L. and Mead, J. F. Chromatographic separation of the plasma lipids. 574.
Finkel, M. P. Relative biological effectiveness of radium and other alpha emitters in CF No. 1 female mice. 494.
Finland, M. and Wilcox, C. Antibiotic combinations and resistance to antibiotics: penicillin with other antibiotics against penicillin-resistant staphylococci. 605.
Fischer, M. A., Coulter, E. P. and Costello, M. J. Effects of x-irradiation and cysteine injection on enzyme systems of rat tissues. 266.
Fishman, M. 205.
Foa, P. P., Magid, E. B., Glassman, M. D. and Weinstein, H. R. Anterior pituitary growth hormone (STH) and pancreatic secretion of glucagon (HGF). 758.
Foldes, F. F. Succinylmonocholine iodide: its enzymatic hydrolysis and neuromuscular activity. 187.
Foley, G. E. Chemical and biological studies on the *1,2*-dihydro-*s*-triazines. Activity in pteroylglutamic acid-*Streptococcus faecalis* 8043 systems. 733; Chemical and biological studies on the *1,2*-dihydro-*s*-triazines. Inhibition of derivatives of pteroylglutamic acid. 740; and Watson, P. L. Chemical and biological studies on the *1,2*-dihydro-*s*-triazines. Development of resistance by *Streptococcus faecalis* 8043. 742.
Folk, J. E. 21.
Fong, J., Nematollahi, J. and Margherita, S. Initial stages of mustard action upon influenzal toxicity and infectivity. 190.
Forbes, G. B., Reid, A., Bondurant, J. and Etheridge, J. Estimation of total body chloride in young infants by radiobromide dilution. 871.
Forbes, J. C. and Petterson, O. M. Effect of intravenous administration of paritol-C on serum lipids of hypercholesterolemic rabbits. 665.
Formal, S. B. 292.
Forman, S., May, L. G., Bennett, A., Kobayashi, M. and Gregory, R. Effects of pressor and depressor agents on pulmonary and systemic pressures of normotensives and hypertensives. 847.
Fountain, J. R., Hutchison, D. J., Waring, G. B. and Burchenal, J. H. Persistence of amethopterin in normal mouse tissues. 369.
Fountaine, J. 360.
Frandsen, A. S. 356.
Friedell, H. L. 618, 748.
Freeman, N. K. 216.
Friedman, M. 212, 318
Fromm, H. J. 329.
Frommhagen, L. H. 838.
Frost, D. V. and Sandy, H. R. Effect of mineral deficiencies on amino acid utilization. Critical role of potassium and phosphorus. 102.
Furth, J. 511.

Gadsden, E. L. and Furth, J. Effect of thyroid hormone on growth of thyrotrophin-secreting pituitary tumors. 511.
Gajdusek, D. C. Suspended cell tissue cultures for study of virus growth kinetics. 621.
Gardner, L. I. Increased plasma 17-ketosteroid concentration in congenital adrenal hyperplasia. 251.
Gaylord, W. H., Jr. 315.
Gebhardt, L. P. and Bachtold, J. G. Reproducible oral infection rates in monkeys with the Mahoney strain (Type I) of poliomyelitis virus. 807.
Geyer, R. P. 384.
Gibson, W. C. and Purkis, V. A. Mosaic photography in the demonstration of human synapses (Boutons Terminaux). 829.
Gillette, R. and Buchsbaum, R. Some effects of desoxycorticosterone glycoside on chick fibroblasts *in vitro*. 30.
Girerd, R. J. 615.
Glassman, M. D. 758.
Glyn, J. 648.
Gochenour, W. S., Jr., 490, 712.
Goerke, J. 212.
Goldberg, B. and Jones, H. W., Jr. Acid phosphatase in human female genital tract, a histochemical and biochemical study. 45.
Gollub, S., Feldman, D., Schechter, D. C., Kaplan, F. E. and Meranze, D. R. Thromboplastinase, a new enzyme which destroys thromboplastin. 858.
Goltz, H. L. 14.
Gomori, G. and Coutinho, H. B. Selective inactivation of esterase by alcohol. 809.
Gong, J. K. 550.
Goodman, J. R., Fountaine, J. and Vincent, J. Cooling of embryonated eggs to produce an LD_{50} for *Coccidioides immitis*. 360.
Gorbman, A. 751.
Gordon, L. E., Miller, C. P. and Hahne, H. J.

Effect of somatotrophic hormone and streptomycin on mice exposed to total body x-irradiation. 85.

Gordon, M. 646.

Granick, S. 525.

Gray, L. F. 487, 625.

Gray, R. S. and Axelrod, A. E. Application of thermistor to measurement of subcutaneous temperatures during hydrothermal injury in the rat. 269.

Green, M. and Stahmann, M. A. Inhibition of mumps virus multiplication by a synthetic polypeptide. 852; Stahmann, M. A. and Rasmussen, A. F., Jr. Protection of embryonated eggs infected with infectious bronchitis or Newcastle disease virus by polypeptides. 641.

Greenwood, D. A., Harris, L. E., Biddulph, C., Bateman, G. Q., Binns, W., Miner, M. L., Harris, J. R., Mangelson, F. and Madsen, L. L. Feeding rats tissues from lambs and butterfat from cows that consumed dot-dusted alfalfa hay. 458.

Gregory, R. 847.

Griffiths, M. and Pace, N. Intracellular phosphorus turnover in the rat liver cell. 771.

Grim, E. Relation between pressure and concentration difference across membranes permeable to solute and solvent. 195.

Grimm, M. 378.

Grimsson, H. 518.

Gross, L. A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinonas in C_3H mice. 414.

Guest, G. M. 327.

Gunnison, J. B. 668.

Gustin, S. T. 143.

Hackett, P. L. Effect of repeated zirconium citrate injections on distribution of plutonium in the rat. 710.

Hahne, H. J. 85.

Halberg, F. and Bock, F. Eosinopenia during last third of pregnancy and after delivery in several stocks of mice. 338.

Halsted, J. A. 226.

Hamilton, J. G. and Scott, K. G. Effect of calcium salt of versene upon metabolism of plutonium in the rat. 301.

Harford, C. G. 678.

Harris, J. R. 458.

Harris, L. E. 458.

Harris, S. C. and Worley, R. C. Human "Analgesic" action of d-amphetamine, amobarbital, acetylsalicylic acid and acetophenetidin, in combination. 515.

Hartsook, E. W., Batchelor, E. and Johnson, B. C. Effect of aureomycin on utilization of vitamin A in the growing male albino rat. 43.

Heiselt, L. R. 722.

Heisler, C. 618.

Helmendach, R. H. 4.

Heming, A. E. and Holtkamp, D. E. Calorigenic and antigoitrogenic actions of L-Triiodothyronine and L-Thyroxine in thyroidectomized and intact rats. 875.

Henkel, D. T. 54.

Hennessey, F. B. 311.

Hernandez, H. H., Peterson, D. W., Chaikoff, I. L. and Dauben, W. G. Absorption of cholesterol-4-C^{14} in rats fed mixed soybean sterols and β-Sitosterol. 498.

Hershberger, L. G., Shipley, E. G. and Meyer, R. K. Myotrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method. 175.

Hess, W. C. and Shaffran, I. P. Effect of cortisone upon formation of liver glycogen from glycine. 804.

Hesselbach, M. L. and Du Buy, H. G. Localization of glycolytic and respiratory enzyme systems on isolated mouse mitochondria. 62.

Heston, W. E. 474.

Hewson, K. 608.

Hill, E. G. 395.

Hine, C. H. 765.

Hisaw, F. L. 509.

Hoag, W. G., Gochenour, W. S., Jr. and Yager, R. H. Leptospiral macroscopic agglutinating antigens. 490; Gochenour, W. S., Jr. and Yager, R. H. Use of baby chicks for isolation of leptospires. 712.

Hoch-Ligeti, C. Influence of low protein diet on distribution of D-amino acid oxidases in rats. 403.

Hoffbauer, F. W., Watson, C. J. and Schwartz, S. Urinary and fecal coproporphyrin excretion in rats: results in normals and castrates. 228; Watson, C. J. and Schwartz, S. Urinary and fecal coproporphyrin excretion in rats. Results in experimental liver damage. 232; Urinary and fecal coproporphyrin excretion in rats. Excretion of injected coproporphyrin. 238.

Hoffman, B., Jr. 249.

Holtkamp, D. E. 875.

Homburger, F. 429.

Hull, R. N. and Lavelle, J. M. Inhibition of cytopathogenic effect of poliomyelitis viruses in tissue culture by antibiotic M-8450. 787.

Hummel, F. P. 126.

Hunter, P. F. 720.

Hutchinson, L. A., Andrews, G. A. and Kniseley, R. M. Influence of cortisone on toxicity of nitrogen mustard in rats. 376.

Hutchison, D. J. 369.

Hyman, C., Stone, A. and Sosnow, M. Application of tissue clearance technics in small animals. 643.

Iglesias, R. 157.

Ingle, D. J., Nezamis, J. E. and Meeks, R. C. Effect of adrenal cortical extract upon work output and glucose tolerance of adrenalectomized-eviscerate rat. 537; Nezamis, J. E. and Morley, E. H. Failure of certain vitamins to affect the survival of the eviscerate rat. 602.

Ishida, T. 318.

Jackson, J. T., Machlin, L. J., Brandenburger, E. A., Kellogg, W. L. and Denton, C. A. Retention of Co 60 labeled vitamin B_{12} in chickens. 221.

Jacobsen, E. M. and Tietz, E. B. Effect of electroshock and electronarcosis on intraocular ovarian and endometrial transplants. 78.

Jaconia, D. 378.

Jawetz, E. 668.

Jenkins, E. M. 88.

Johansson, K. R. Response to and assay of vitamin B_{12} by a mutant of *Escherichia coli.* 448.

Johnson, B. C. 43.

Johnson, D. H. 790.

Jones, H. W., Jr. 45.
Jungeblut, C. W., Hoffman, B., Jr. and Verlinde, J. D. Absorption *in vitro* of poliomyelitis virus on human erythrocytes. 249.
Junqueira, L. C. U. 455.

Kandel, A. 408.
Kaplan, F. E. 858.
Kay, E. R. M. 321.
Kazlowski, J. 253.
Kelley, V. C. 722.
Kellogg, W. L. 221.
Kent, J. F. 811.
Kerby, G. P. Chromatographic separation of heparin and chondroitin sulfate. 263; and Eadie, G. S. Inhibition of lysozyme by heparin. 111.
Kerner, E. and Westerfeld, W. W. Effect of diet on rats of alcohol oxidation by the liver. 530.
Kidder, G. W. 586.
Kilham, L. and Woke, P. A. Laboratory transmission of fibromas (Shope) in cottontail rabbits by means of fleas and mosquitoes. 296.
King, M. E. Effect of phenylbutazone (butazolidin) on renal excretion of P-aminohippurate and thiosulfate in the dog. 827.
Kinsell, L. W., Balch, H. E. and Michaels, G. D. Accentuation of human diabetes by "pituitary growth hormone." 683.
Kirkham, W. R. and Turner, C. W. Nucleic acids of the mammary glands of rats. 123.
Kirtley, W. R., Waife, S. O. and Peck, F. B. Effect of glucagon in stable and unstable diabetic patients. 387.
Kleeman, C. R. 660.
Klein, S. J. and Adams, J. M. Experimental poliomyelitis in mice with human gamma globulin. 308.
Kniseley, R. M. 376.
Knobil, E. Effects of hypophysectomy, ACTH, cortisone and estradiol on β-glucuronidase activity of adrenal gland in the rat. 769.
Knobloch, W. H., Jr. 75.
Kobayashi, M. 847.
Kodama, J. K. 765.
Kokko, U. P. 200.
Kornblum, J. 527.
Kowalski, H. J., Reynolds, W. E. and Rutstein, D. D. Electrolyte and water responses of normal subjects during cortisone administration. 795.
Kraemer, D. M. 209.
Kuh, E. 398, 438.
Kull, F. C. 378.
Kulp, W. L. 599.
Kun, E. Occurrence of hexose-6-phosphate dehydrogenase in chorioallantoic membrane of the chick embryo. 532.
Kutsky, R. J. Stimulating effect of nucleoprotein fraction of chick embryo extract on homologous heart fibroblasts. 390.

Labriola, J. Effects of caesarean delivery upon maternal behavior in rats. 556.
Lagen, J. B. 384.
Laskowski, M. 686.
Lasser, R. P. and Loewe, L. Valve-like action of segment of aorta communicating between chambers of left atrium and left ventricle. 401.
Laster, W. R., Jr. 1.
Lavelle, J. M. 787.
Lavietes, P. H. 548.
Lavik, P. S., Leonards, J. R., Buckaloo, G. W., Heisler, C. and Friedell, H. L. Ineffectiveness of *in vivo* dialysis in prolonging life in x-irradiated dogs. 618.
Lazarus, S. S. 151.
Leonards, J. R. 618.
Le Page, G. A. Effects of chloramphenicol on incorporation of glycine-2-C14 into mammalian tumor cell proteins and purines. 724.
Levenson, S. M. 91.
Levi, C. 143.
Lewis, F. J. 639.
Li, C. P. and Schaeffer, M. Further modification of the mouse adapted type III poliomyelitis virus. 706.
Libby, R. L. 226.
Liener, I. E. and Rose, J. E. Soyin, a toxic protein from soybean, immunochemical properties. 539.
Lilly, D. M., Sterbenz, F. J. and Tarantola, V. Growth promotion in carnivorous protozoa by substituted purines. 434.
Lipschutz, A. 157.
Little, R. C., Strain, S. F., Jr. and Schaefer, R. D. Effect of initial length on the work of the cardiac atrium. 258.
Localio, S. A. 798.
Loefer, J. B. 54.
Loewe, L. 401.
Love, R. and Frommhagen, L. H. Histochemical studies on the clam *Mactra Solidissima.* 838.

MacDonald, E. and Bahner, C. T. Hafnium complexes for biological investigation. 801.
Machlin, L. J. 221.
Mack, H. P. 264.
MacKay, M. 798.
Mackler, B. and Guest, G. M. Effects of insulin and glucose on utilization of fructose by isolated rat diaphragm. 327.
Mac Vicar, R. 571.
Madsen, L. L. 458.
Magee, D. F. 110.
Magid, E. B. 758.
Mangelson, F. 458.
Marcus, S. and Donaldson, D. M. Suppression of normal bactericidal action of rabbit serum following whole body x-irradiation. 184.
Mardones, E. 157.
Margherita, S. 190.
Marston, R. Q., Ruth, H. J. and Smith, W. W. Role of host defenses in streptomycin treatment of x-irradiated mice. 289.
Martin, C. J. and Axelrod, A. E. A modified method for determination of hydroxyproline. 461; 463.
Marx, W., Gustin, S. T. and Levi, C. Effects of thyroxine, thyroidectomy and lowered environmental temperature on incorporation of deuterium into cholesterol. 143.
Mason, R. C. 790.
Masson, G. M. C., Del Greco, F., Corcoran, A. C. and Page, I. H. Factors influencing renin diuresis and proteinuria. 631.
Maurer, P. H. Dextran, an antigen in man. 879; Dixon, F. J. and Talmage, D. W. A quantitative comparison of antibody produced by x-radiated and normal rabbits. 163; 287.

May, L. G. 847.
Mayer, R. L., Grimm, M., Jaconia, D. and Kull, F. C. Role of lipids in formation of yellow pigment from p-aminobenzoic and p-aminosalicylic acids. 378.
McClure, F. J. and Folk, J. E. Skim milk powders and experimental rat caries. 21.
McConnell, K. P., Portman, O. W. and Rigdon, R. H. Intravascular life span of the duck red blood cell as determined by radioactive selenium. 140.
McCulloch, W. S. 306.
McDougall, H. A. 798.
McEwen, C. 648.
McGhee, R. B. 349.
McHenry, E. W. 781.
Mead, J. F. 574.
Meeks, R. C. 537.
Mefferd, R. B., Jr., Henkel, D. T. and Loefer, J. B. Effect of piromen on survival of irradiated mice. 54.
Meites, J. 872.
Melnick, J. L. and Gaylord, W. H., Jr. Problems with spontaneous ectromelia (mouse pox) in a virus laboratory. 315.
Meranze, D. R. 858.
Merritt, J. B. 269.
Meyer, K. F. and Eddie, B. Characteristics of a psittacosis viral agent isolated from a turkey. 99.
Meyer, L. M., Suarez, R. M., Jr., Buso, R., Sabater, J. and Suarez, R. M. Oral administration of Co^{60} vit. B_{12} in tropical sprue and hepatic cirrhosis and diarrhea. 681.
Meyer, R. K. 175.
Michaels, G. D. 683.
Migliarese, J. 398.
Milhorat, A. T. 57, 637.
Miller, B. F. 352.
Miller, C. Reversible inhibition of cell division and enlargement in plant tissues by 2,6-diaminopurine. 561.
Miller, C. P. 85.
Miller, H. and Kraemer, D. M. Aortic albuminoid titration and swelling curves from persons differing in age and degree of atherosclerosis. 209.
Miller, L. L. 483.
Milman, A. E., Silides, D. N. and Milhorat, A. T. Nitrogen metabolism of isolated muscle of normal and vitamin E-deficient hamsters. 637.
Miner, M. L. 458.
Minz, D., Remond, A. G. and McCulloch, W. S. Local effects of epinephrine on the cerebral cortex. 306.
Mirand, E. A., Reinhard, M. C. and Goltz, H. L. Development of sarcomas in Marsh-albino mice following injection of desoxycorticosterone acetate in sesame oil. 14.
Mitchell, M. L. and Girerd, R. J. Effect of growth hormone and antibiotics upon nitrogen mustard treated rats. 615.
Mitchell, W. G. Antagonism of toxicity of vanadium by ethylenediamine tetraacetic acid in mice. 346.
Molomut, N. 326.
Mommaerts, E. B., Beard, D. and Beard, J. W. Screening of chicks with erythromyeloblastic leukosis for plasma activity in dephosphorylation of adenosine triphosphate. 479.
Mooser, H. and Weyer, F. Experimental infection of *Macacus rhesus* with *Rickettsia quintana* (trench fever). 376.
Morgan, A. F. 655.
Morley, E. H. 602.
Morse, M. S., Kodama, J. K. and Hine, C. H. Cholinesterase-inhibiting properties of two vinyl-substituted phosphates. 765.
Morton, H. E. 65.
Mota, I., Beraldo, W. T. and Junqueira, L. C. U. Protamine-like property of compound 48/80 and stilbamidine and their action on mast cells. 455.
Mueller, J. F. Pathologic physiology of mammalian blood platelet utilizing P^{32} tagged rabbit platelets. 557.
Mukherjee, A. K. 133.
Murray, J. E. 352.
Murray, R. and Ratner, F. Safety of immune serum globulin with respect to homologous serum hepatitis. 554.

Nagler, F. P. 714.
Nath, M. C., Belavady, B., Sahu, V. K. and Chitale, R. P. Biosynthesis of vitamin C: a new precursor. 39.
Nematollahi, J. 190.
Neve, R. A. 566.
Nezamis, J. E. 537, 602.
Nichol, C. A. On the metabolic alteration of pteroylglutamic acid. 167; Zakrzewski, S. F. and Welch, A. D. Resistance to folic acid analogues in a strain of *Streptococcus faecalis.* 272.
Nichols, A. V. 216.
Nichols, C. W., Jr., Siperstein, M. D. and Chaikoff, I. L. Effect of dihydrocholesterol (cholestanol) administration on plasma cholesterol and atherosclerosis in the rabbit. 756.
Norman, A. 71.
North, W. C. 825.
Northrup, D. W. 98.
Noyes, W. F. A simple technic for demonstrating plaque formation with virus of vaccinia. 426.
Nungester, W. J. and Van Halsema, G. Reaction of certain phytoagglutinins with Flexner-Jobling carcinoma cells of the rat. 863.

Obrinsky, W. 502.
Oppenheimer, J. H. and Riester, W. H. Influence of cortisone on leptomeningeal reaction induced by talc. 844.
Orbison, J. L. and Peters, E. Failure of rutin and catechin to affect vascular lesions of experimental hypertension or hypersensitivity. 173.
Ord, M. G. and Stocken, L. A. Sex differences in effect of radiation and fluoracetate poisoning on liver metabolism. 695.
Ormsbee, R. A. Effect of erythromycin, thiocymetin, and three other antibiotics on *Leptospira icterohemorrhagiae* in the chick embryo. 815.
Ozawa, G. 781.

Pace, N. 771.
Paff, G. H. and Stewart, R. Free wandering cells and cortisone. 591.
Page, I. H. 631.
Palsson, P. A. and Grimsson, H. Demyelination in lambs from ewes which feed on seaweeds. 518.

Park, H. D. 662.
Parker, R. F. 577.
Parker, R. P. 398, 438.
Parkins, W. M. 146.
Partridge, R. 398.
Paschkis, K. E. 181.
Paterson, P. Y., Pond, W. L., Warren, J. and Weil, M. L. Encephalitogenic properties of crude and protamine treated rabbit and mouse brain suspensions. 278.
Patt, H. M., Blackford, M. E. and Drallmeier, J. L. Growth characteristics of the Krebs ascites tumor. 520.
Patterson, J. W. Prevention of dehydroascorbic acid diabetes by atropine. 850.
Paulissen, L. J. 205.
Peanasky, R. J. and Laskowski, M. Identification of trypsinogen in the lipotropic fraction. 686.
Peck, F. B. 387.
Peck, G. C., Mack, H. P. and Figge, P. H. J. A study of the effect of hematoporphyrin on hypophyseal-adrenal relationships. 264.
Pelczar, M. J., Jr. 284.
Perlman, P. L. and Cassidy, J. W. Influence of nitrogen intake on nitrogen-retaining action of testosterone propionate. 674.
Perlmutt, J. H., Parkins, W. M. and Vars, H. M. Response of dogs to intravenous administration of polyvinylpyrrolidone and its monomer. 146.
Peters, E. 173.
Peterson, D. W. 498.
Petterson, O. M. 665.
Pierce, W. A., Jr., Steele, R. H. and White, A. G. C. A colorimetric method for hyaluronic acid estimation. 373.
Pirozynski, W. J. 335.
Pollard, M. and Bussell, R. H. An antineoplastic factor in spleens of mice previously inoculated with methylcholanthrene. 671.
Pond, W. L. 278.
Pope, L. S. 571.
Portman, O. W. 140.
Powell, H. M. and Culbertson, C. G. Action of an antiviral mold filtrate against MEF1 poliomyelitis virus in mice. 161.
Praglin, J. 155.
Purkis, V. A. 829.

Quick, A. J. 453.
Quinn, M., Bass, D. E. and Kleeman, C. R. Effect of acute cold exposure on serum potassium, and magnesium and the electrocardiogram in man. 660.

Rachmilewitz, M. 50.
Raffucci, F. L., Lewis, F. J. and Wangensteen, O. H. Hypothermia in experimental hepatic surgery. 639.
Rall, D. P. and North, W. C. Consideration of dose/weight relationships. 825.
Ralli, E. P. 588.
Ramirez, H., Iglesias, R., Mardones, E. and Lipschutz, A. Mechanism of ovarian control of the hypophyseal gonadotrophic function in the guinea-pig. Experiments with intrasplenic grafts. 157.
Randall, C. C. and Turner, D. J. Cultivation of yeast in Earle's "L" strain mouse cells *in vitro*. 584.
Rasmussen, A. F., Jr. 641.
Ratner, F. 554.
Reagan, R. L., Stewart, M. T. and Brueckner, A. L. Study of nine rabies street virus strains in the Syrian hamster. 793.
Reaven, G. 281.
Reeve, R. H. 113.
Register, U. D. 708.
Reid, A. 871.
Reinhard, M. C. 14.
Remond, A. G. 306.
Reynolds, W. E. 795.
Richards, J. B. 628.
Richert, D. A. and Westerfeld, W. W. Some interrelations of dietary protein, molybdenum, riboflavin and calories on liver and intestinal xanthine oxidase. 726.
Richmond, V. 75.
Riester, W. H. 844.
Rigdon, R. H. Fibroma arising from feather follicle of duck following local application of methylcholanthrene to skin. 34; 140.
Riggs, T. R. 697.
Robbins, E. D., Burton, S., Goerke, J. and Friedman, M. Cholesterol and cholate concentration of perfusate and bile of isolated liver preparation. 212.
Roberts, E. and Hunter, P. F. Inhibition of utilization of aspartic acid for growth of *Leuconostoc mesenteroides* P-60. 720.
Robinson, H. J., Mason, R. C., Johnson, D. H. and Smith, A. L. Effect of adrenocorticotropic hormone on pneumococcal infections in rabbits. 790.
Romoser, G. L., Shorb, M. S. and Combs, G. F. Effect of orally administered penicillin-resistant microorganisms on growth of chicks. 17.
Rooney, D. 544.
Rose, J. E. 539.
Rosen, H. and Levenson, S. M. Non-protein nitrogen changes in serum and plasma of rats following thermal injury. 91.
Rosenbaum, H. E. and Harford, C. G. Effect of fatigue on susceptibility of mice to poliomyelitis. 678.
Rosenbaum, P. and Obrinsky, W. Effect of cortisone on diphtheria intoxication and the Schick test in guinea pigs. 502.
Rosenberg, S. 748.
Rosenkrantz, H. and Milhorat, A. T. Arginase activity in vitamin E-deficient rats. 57.
Rubin, A. A. and Gordon, M. Effects of alpha-estradiol benzoate and methyl testosterone upon the Platyfish, *Xiphophorus maculatus* skeleton. 646.
Rubin, B. L. and Dorfman, R. I. Bioassay of parathyroid hormone. 223.
Rudzinska, M. A. and Granick, S. Protoporphyrin production of *Tetrahymena geleii*. 525.
Rugh, R. Selective sterilization of young female mice by radioiodine transmitted through the milk. 762; and Wang, S. C. Protection against radiation lethality: effect of β-mercaptoethylamine. 411.
Ruth, H. J. 289.
Rutstein, D. D. 795.

Sabater, J. 681.
Sahu, V. K. 39.
Saleeby, R. G. 423.

Samuels, P. B. 335.

Sanders, P. 259.

Sandy, H. R. 102.

Sawyer, C. H. and Everett, J. W. Priming the anestrous cat for reflex discharge of pituitary ovulating hormone. 820.

Sawyer, J. 281.

Sayers, G. 729.

Scaletti, J. V., Kulp, W. L. and Cleverdon, R. C. Effect of terramycin on fecal microflora of rats. Therapeutic level of terramycin. 599.

Schabel, F. M., Jr., Laster, W. R., Jr., Brockman, R. W. and Skipper, H. E. Observations on antiviral activity of netropsin. 1.

Schaefer, R. D. 258.

Schaeffer, M. 706.

Schales, O. Effect of additions to ACD blood on erythrocyte preservation. 593.

Schechmeister, I. L., Paulissen, L. J. and Fishman, M. Sublethal total body x-radiation and susceptibility of mice to *Salmonella enteritidis* and *Escherichia coli*. 205.

Schechter, D. C. 858.

Schenck, R. 52.

Schilling, R. F. and Deiss, W. P. Intrinsic factor studies I. Paper electrophoresis of mixture of gastric juice and radioactive vitamin B_{12}. 506; and Eisenhauer, E. R. Mechanical fragility of erythrocytes from leukemic patients. 214.

Schreder, J. S. and Rooney, D. Excretion of 3-(3-amino-2,4,6-triiodophenyl)-2-ethyl-propanoic acid (telepaque) by man. 544.

Schwartz, H. S. 500.

Schwartz, S. 232, 228, 238.

Schwartz, T. B. Proteolytic activity and protein catabolism of rat diaphragm in hemorrhagic shock. 362; and Engel, F. L. Rat plasma aminopeptidase activity in hemorrhagic shock. 568.

Scott, K. G. 301.

Scull, E. 648.

Seeger, D. R. 398, 438.

Segal, H. L. and Miller, L. L. Tubeless gastric analysis by use of insoluble salts with exogenous ions. 483.

Seibert, F. B. 535.

Seifter, J., Baeder, D. H., Beckfield, W. J., Sharma, G. P. and Ehrich, W. E. Effect of hyaluronidase on experimental hypercholesterolemia in rabbits and rats and atheromatosis in rabbits. 468.

Seligmann, E. Virulence enhancement of *Candida albicans* by antibiotics and cortisone. 778.

Shaffran, I. P. 804.

Sharma, G. P. 468.

Sherwood, D. H., Hill, E. G. and Sloan, H. J. L-lyxoflavin in the starting diets of turkeys and chickens. 395.

Shetlar, C. L. 75.

Shetlar, M. R., Knobloch, W. H., Jr., Richmond, V., Shetlar, C. L. and Everett, M. R. Effect of fever on serum polysaccharide level. 75.

Shettles, L. B. 692.

Shinowara, G. Y., Waite, J. H. and Saleeby, R. G. Plasma coagulation factors in acute pancreatitis. 423.

Shipley, E. G. 175.

Shorb, M. S. 17, 284.

Shore, B., Nichols, A. V. and Freeman, N. K. Evidence for lipolytic action by human plasma obtained after intravenous administration of heparin. 216.

Shuman, C. R. 323.

Siemienski, J. 160.

Silides, D. N. 637.

Silverberg, R. J. 200.

Siperstein, M. D. 756.

Skipper, H. E. 1.

Sloan, H. J. 395.

Smith, A. L. 790.

Smith, P. F. and Morton, H. E. Nature of growth inhibitor in some mammalian sera for pleuropneumonia-like organisms of human origin. 65.

Smith, W. W. 289.

Sonnenschein, R. R. The tetracaine flare: differences in actions of procaine and tetracaine on peripheral nerve. 831.

Sosnow, M. 643.

Spain, D. M. and Molomut, N. Failure of somatotrophic hormone (STH) to counteract cortisone action on wounds in mice. 326.

Spilman, W. 292.

Stahmann, M. A. 641, 852.

Stanfield, A. B., Brodie, E. C. and Yeoman, E. E. Butazolidin plasma concentration and urinary excretion in normal individuals and in patients with rheumatoid spondylitis. 254.

Starr, P. and Schenck, R. Effect of oral thyroxine & tri-iodo-thyronine on radioactive iodine uptake & serum protein bound iodine in normal human subjects. 52.

Steele, R. H. 373.

Stefanini, M. 105, 311.

Steigman, A. J., Kokko, U. P. and Silverberg, R. J. Mack virus-serum and gamma globulin neutralization of unidentified agent isolated from suspected non-paralytic poliomyelitis. 200.

Sterbenz, F. J. 434.

Stevens, C. E. 181.

Stewart, M. T. 793.

Stewart, R. 591.

St. George, S., Friedman, M. and Ishida, T. Intracellular distribution of digitoxin. 318.

Stocken, L. A. 695.

Stone, A. 643.

Storassli, J. P., Rosenberg, S. and Friedell, H. L. Effect of food and water on thyroid uptake of radioiodine (I^{131}) in rats. 748.

Storer, J. B. and Sanders, P. Effect of pituitary extracts on radiation-induced testicular atrophy in rats. 259.

Strain, S. F., Jr. 258.

Suarez, R. M. 681.

Suarez, R. M., Jr. 681.

Swendseid, M. E., Halsted, J. A. and Libby, R. L. Excretion of cobalt^{60}-labeled vitamin B_{12} after total gastrectomy. 226.

Swim, H. E. and Parker, R. F. Method for establishing human cells in culture: susceptibility to poliomyelitis after prolonged cultivation. 577.

Sydnor, K. L. and Sayers, G. Biological half-life of endogenous ACTH. 729.

Tabenkin, B., Cooperman, J. M., Drucker, R. E. and Jenkins, E. M. Effect of feeding several sulfonamide and related compounds on growth of chicks. 88.

Talmage, D. W. 163.
Tappan, D. V., Boldt, R. E. and Elvehjem, C. A. Unidentified factors capable of reducing stress in iodinated protein-fed rats. 135.
Tarantola, V. 434.
Taylor, A. and Carmichael, N. Egg cultivated tumor protects embryo against vaccinia virus. 676.
Thompson, J. C. and Vars, H. M. Biliary excretion of cholic acid and cholesterol in hyper-, hypo-, and euthyroid rats. 246.
Tietz, E. B. 78.
Trager, W. and McGhee, R. B. Inhibition of chicken tumor I by plasma from chickens infected with an avian malaria parasite. 349. ,
Turner, C. W. 123.
Turner, D. J. 584.
Turner, R. 323.

Ungar, G. and Hummel, F. P. Inhibition of aldehyde oxidation by adrenergic and adrenolytic substances. 126.

Van Halsema, G., 863.
Van Liere, E. J., Fang, H. S. and Northrup, D. W. Effect of semi-starvation on propulsive motility of the small intestine. 98.
Van Wagenen, G. 548.
Vars, H. M. 146, 246.
Velardo, J. T., Hisaw, F. L. and Dawson, A. B. Growth of deciduomata in adrenalectomized rats. 509.
Veltre, F. A., Shorb, M. S. and Pelczar, M. J., Jr. Nutritional requirements of *Lactobacillus bifidus* isolated from poults and chicks. 284.
Vera, C. 784.
Verlinde, J. D. 249.
Villarreal, R. Pepsin secretion and synthesis *in vitro*. 817
Vincent, J. 360.
Volk, B. W. and Lazarus, S. S. Metabolic effects of growth hormone in fasting, phlorhizinized and adrenalectomized rats. 151.
Von Kaulla, K. N. and Shettles, L. B. Relationship between human seminal fluid and the fibrinolytic system. 692.

Wagner, R. R. Production of fever by influenzal viruses. Tolerance to mumps and influenza pyrogens. 612.
Waife, S. O. 387.
Waite, J. H. 423.
Wang, S. C. 411.
Wangensteen, O. H. 639.
Wannamacher, R. 398.
Waring, G. B. 369.
Warren, J. 278.
Watson, C. J. 228, 232, 238.
Watson, P. L. 742.
Weil, M. L. 278.
Weinstein, H. R. 758.
Weinstein, L. Antigenic dissimilarity of streptokinases. 689.
Welch, A. D. 272.
Weller, T. H. Serial propagation *in vitro* of agents producing inclusion bodies derived from varicella and herpes zoster. 340.
Wemyss, C. T., Jr. 352.
Westerfeld, W. W. 530, 726.
Weyer, F. 699.
White, A. G. C. 373.
White, S. G., Aggeler, P. M. and Emery, B. E. Plasma thromboplastin component (PTC) potency of plasma fractions. 69; Lagen, J. B. Aggeler, P. M. and Geyer, R. P. Effect of soybean phosphatide on blood coagulation defect following total body x-irradiation in the dog. 384.
Whitehair, C. K. 571.
Wilcox, C. 605.
Williamson, M. B. and Fromm, H. J. Utilization of sulfur amino acids during healing of experimental wounds. 329.
Wilson, H., Glyn, J., Scull, E., McEwen, C. and Ziff, M. Rate of disappearance and metabolism of hydrocortisone and cortisone in the synovial cavity in rheumatoid arthritis. 648.
Wilwerth, A. M. and Meites, J. Effects of cortisone on thiamine-deficient young rats. 872.
Winter, J. L. Development of antibodies in children convalescent from whooping cough. 866.
Wohl, M. G., Shuman, C. R. and Turner, R. Effect of mercurials on thiamine excretion in patients with congestive heart failure. 323.
Woislawski, S. Ionization constants of basic dyes in relation to gastric secretion. 130.
Woke, P. A. 296.
Worley, R. C. 515.

Yager, R. H. 490, 712.
Yeoman, E. E. 254.

Zakrzewski, S. F. 272.
Ziff, M. 648.

SUBJECT INDEX

VOLUME 83

(The numerals indicate the page)

Acid, amino, oviduct. 697.
    sulfur, wound healing. 329.
  ascorbic dehydro, metabolic effects. 133.
  cholic, excretion, thyroid influence. 246.
  fatty, metabolism, shock. 608.
  glutamic, oxidation, heart. 367.
  hyaluronic, assay. 373.
  nucleic, mammary gland. 123.
    -ribo, phosphorylation. 321.
  uric, excretion, phenylbutazone effect. 603.
Adenosine triphosphate, dephosphorylation, erythromyeloblastic leukosis. 479.
Adrenal, blood, venous, sodium-retaining substance. 628.
  cortex, DDD. 59.
  hematoporphyrin. 264.
  hyaluronidase reaction. 588.
  hyperplasia, 17-ketosteroid plasma. 251.
Adrenalectomy, deciduomata growth. 509.
Adrenergy, aldehyde oxidase inhibition. 126.
Albumen, serum, half life. 287.
Alcohol oxidation, liver, diet, effect. 530.
Alpha emitters, effect, CF1 mice. 494.
Analgesia, drug combination. 515.
Anesthesia, body temperature. 4.
  thiopental, fat feeding effect. 110.
Antibiotic, aureomycin, alloxan diabetes, weight response. 50.
    vitamin A, utilization. 43.
  blood coagulation. 160.
  Candida, virulence enhancement. 778.
  chloramphenicol, glycine metabolism. 724.
  combinations, staphylococci resistance. 605.
  erythromycin and carbomycin combined, action. 668.
    leptospira. 815.
  netropsin, antiviral. 1.
  nitrogen mustard. 615.
  penicillin, growth effect. 17.
    susceptibility, *M. pyogenes.* 527
  poliomyelitis, cytopathogenic inhibition. 787.
    virus chemoprophylaxis. 161.
  streptomycin, X-radiation. 85.
    X-ray, host defenses. 289.
  terramycin, fecal flora. 599.
  thiocymetin, leptospira. 815.
Antibody, complement fixation, measurement micro. 113.
  Endamoeba histolytica. 811.
  hemagglutinogen, primates. 701.
  production, X-radiation effect. 163.
Aorta, albuminoid swelling curves. 209.
Arthritis, rheumatoid, cortisone and hydrocortisone, disappearance and metabolism. 648
  phenylbutazone. 603.
Aspartic acid utilization, *Leuconostoc mesenteroides* growth. 720.

B. diphtheriae, intoxication, cortisone effect. 502.
Bacteria, *E. coli,* vitamin B_{12} assay. 448.
  fecal, terramycin effect. 599.
  genetics, phage lysates. 292.
  growth effect, penicillin. 17.
  M. pyogenes, penicillin susceptibility. 527.
  pleuropneumonia-like, inhibitor. 65.
  pneumococcus, ACTH influence. 790.
  staphylococcus, resistance, antibiotic combinations. 605.
  streptococcus, type culture, isolation. 579.
Bactericidal action, X-radiation effect. 184.
Bacteriophage lysates, genetic transfer. 292.
Bacterium, *L. bifidus,* nutrition. 284.
  L. leichmannii, molybdenum toxicity. 487.
Behavior, rats, caesarean delivery. 556.
Benzimidazol, poliomyelitis. 408.
Blood, adrenal, venous, sodium-retaining substance. 628.
  cholesterol, paritol-C effect. 665.
  coagulation, antibiotic influence. 160.
    pancreatitis. 423.
    X-ray, phosphatide effect. 384.
  electrophoresis, human. 429.
  eosinophils, pregnancy. 338.
  erythrocyte, duck, life span. 140.
    fragility, leukemia. 214.
    preservation, ACD blood. 593.
  erythropoiesis stimulation, "anemic" serum effect. 548.
  flowmeter. 155.
  globulin, immune serum, safety, serum hepatitis. 554.
  hemophilia, protein electrophoresis. 311.
  leukemia, erythrocyte fragility. 214.
  leukosis, erythromyeloblastic, adenosine triphosphate dephosphorylation. 479.
  lipids, Paritol-C effect. 665.
    separation, chromatography. 574.
  plasma fractions, PTC. 69.
    thromboplastin, properties. 105.
  platelet, physiology, P^{32}. 557.
  polysaccharide, fever effect. 75.
  pressure, pulmonary and systemic, pressor and depressor agents. 847.
  vessel, atheroma, cholesterol, hyaluronidase effect. 468.
    lesions, experimental, flavonol influence. 173.
Brain, stimulation, epinephrine. 306.
  suspension, encephalitogenic. 278.
Breast transfer, vitamin A and carotene. 571.
Brucellosis, diagnosis, surface fixation. 36.
Burn, non-protein nitrogen blood levels. 91.
  proteolytic enzyme and collagen. 463.
Butazolidin, p-aminohippurate and thiosulfate excretion. 827.
  urine and plasma. 254.
Butazone phenyl, uric acid excretion. 603.

Calcification, metastatic, toluidine blue effect. 281.
Candida, virulence enhancement, antibiotics and cortisone. 778.
Carotene, placenta and mammary transfer. 571.
Cell division and enlargement, diaminopurine inhibition. 561.
Chlorides, total body, determination, radiobro-

mide. 871.
Chloroform, mice, C_3H. 474.
Cholate production, liver. 212.
Cholesterol, atheroma, hyaluronidase effect. 468.
  C^{14} absorption, sterols, rat diet. 498.
  dihydro, plasma cholesterol and atherosclerosis relation. 756.
  excretion, thyroid influence. 246.
  metabolism. 71.
  production, liver. 212.
  thyroid metabolism. 143.
Choline, iodide succinylmono, activity and hydrolysis. 187.
Clam, histochemistry. 838.
Collagen, proteolytic enzyme, burn. 463.
Convulsions, suppression, nitrogen hyperoxic. 546.
Coproporphyrin, excretion. 238.
  liver damage. 232.
  normal. 228.

DCA sesame oil, sarcoma production. 14.
DDD, adrenal cortex effect. 59.
Death, body temperature. 4.
Deciduoma, adrenalectomy effect. 509.
Defense, host, X-ray and streptomycin effect. 289.
Dextran, antigenicity. 879.
Diabetes, alloxan, aureomycin effect. 50.
  dehydroascorbic acid, atropine, prevention. 850.
  glucagon. 387.
  pituitary growth hormone, influence. 683.
Diet, hyaluronidase reaction. 588.
Digitoxin, distribution, intracellular. 318.
Diuretic, mercurial, thiamine excretion. 323.
Dose/weight relationships. 825.
Dyes basic, ionization constants, gastric secretion. 130.

Electrocardiogram, cold effect. 660.
Endamoeba histolytica, antiserum immobilization. 811.
Endometrium transplant, electroshock and electronarcosis. 78.
Enzyme aldehyde oxidase, adrenergic effect. 126.
  aminopeptidase, plasma, hemorrhagic shock. 568.
  arginase, vitamin E deficiency. 57.
  carboxypeptidase, inhibitor, X-ray effect. 6.
    tissue. 10.
  cholinesterase inhibition, vinyl-substituted phosphates. 765.
  dehydrogenase, hexose-6-phosphate, embryo, chick. 532.
  esterase, alcohol inactivation. 809.
  glucuronidase β activity, hypophysectomy, ACTH, cortisone, and estradiol effects. 769.
  glycolytic, brain mitochondria. 62.
  hyaluronidase, hypercholesterolemia and atheroma. 468.
  lysozyme, heparin. 111.
  oxidase, d-amino, diet influence. 403.
    cytochrome, zinc effect. 625.
  pepsin, secretion and synthesis. 817.
  phosphatase acid, genital tract. 45.
  proteolytic, collagen, burn. 463.
  respiratory, brain mitochondria. 62.
  thromboplastinase. 858.
  tissue, X-ray effects. 266.
  trypsin, growth effect. 269.
    hypochlorite treated, properties. 242.
  trypsinogen, identification. 686.
  tyrosinase, pro, sulfhydryl groups. 717.
    sulfhydryl groups. 717.
  xanthine oxidase, diet, influence. 726.

Fatigue, poliomyelitis susceptibility. 678.
Fever, blood polysaccharide level. 75.
Fibrinolysis, semen relationship. 692.
Fibroblast, Doca glycoside effect. 30.
  stimulation, nucleoprotein effect. 390.
Fibroma rabbit, insect transmission. 296.
Flavonol, experimental hypertension and hypersensitivity. 173.
Fluoracetate, liver metabolism, sex difference. 695.
Fructose metabolism, diaphragm, insulin effect. 327.
Fungus *coccidioides,* LD_{50}. 360.

Globulin-gamma, poliomyelitis. 308.
Glucagon, diabetes. 387.
Glycine metabolism, cortisone influence. 804.
Gout, phenylbutazone. 603.
Granuloma, talc, meningeal, cortisone effect. 844.
Growth inhibitor, trypsin influence. 269.
  promotion, protozoa, purines effect. 434.
  sulfonamide influence. 88.

Hafnium complexes. 801.
Hay, alfalfa, dot-dusted, rat tissues. 458.
Heart, auricle, work performed. 258.
  mitral orifice, valve artificial. 401.
Hematoporphyrin, pituitary-adrenal stimulation. 264.
Heparin, chondroitin sulfate, separation, chromatography. 263.
  lysozyme inhibition. 111.
  plasma lipolysis. 216.
Hormone, ACTH, adrenal β glucuronidase activity. 769.
    B_6 deficiency, eosinophile response. 655.
    endogenous, half-life. 729.
    pneumococcal infection. 790.
  adrenal cortex, work output and glucose tolerance. 537.
  androgen, myotrophic activity. 175.
  antidiuretic, inactivation, pregnancy serum. 784.
  corticotropin, extraction. 334.
  cortisone, adrenal β glucuronidase activity. 769.
    Candida, virulence enhancement. 778.
    chick embryo. 31.
    diphtheria intoxication and Schick test. 502.
    electrolyte and water balance. 795.
    free wandering cells. 591.
    liver glycogen, glycine. 804.
    mast cell. 335.
    meningeal talc granuloma. 844.

    - metabolism, liver. 27.
      - synovial cavity. 648.
    - nitrogen mustard toxicity. 376.
    - somatotrophic hormone effect. 326.
    - thiamine deficiency. 872.
    - thyroid pituitary interaction. 181.
  - Doca glycoside, fibroblast. 30.
  - epinephrine, B_6 deficiency, eosinophile response. 655.
    - brain effects. 306.
  - estradiol, adrenal β glucuronidase activity. 769.
    - benzoate, skeleton fish. 646.
  - growth, metabolic effects. 151.
    - nitrogen mustard. 615.
  - hydrocortisone, metabolism, synovial cavity. 648.
  - 17-hydroxycorticosteroids, scurvy. 722.
  - insulin, fructose metabolism. 327.
  - ketosteroid 17-, adrenal hyperplasia. 251.
  - methyl testosterone, skeleton fish. 646.
  - parathyroid, assay. 223.
  - pituitary, gonadotrophic, ovarian control. 157.
    - growth, diabetes accentuation. 683.
      - liver enzymes. 781.
      - pancreatic glucagon. 758.
    - ovulating, reflex discharge. 820.
    - somatotrophic cortisone. 326.
    - testicular atrophy radiation induced. 259.
  - somatotrophic, X-radiation. 85.
  - testosterone, nitrogen metabolism. 674.
  - thyroid, pituitary tumors, thyrotrophin-secreting. 511.
  - thyroxine, mast cell. 335.
- **Hyaluronidase,** diet and adrenal influence. 588.

- **Infection,** X-ray, effect. 205.
- **Intestine,** motility, starvation effect. 98.
- **Iodine** utilization, thyroid, fish. 751.
- **Isotope,** P^{32}, TEPA, distribution. 398.

- **Ketone,** metabolism, shock. 608.
- **Kidney,** homotransplant, serum complement. 352.

- **Leptospira,** antigen, agglutinating. 490.
  - isolation, chick. 712.
  - icterohemorrhagiae, antibiotic effect. 815.
- **Lipids,** PABA and PAS oxidation. 378.
- **Lipoprotein,** lipolysis, heparin. 216.
- **Liver,** cholesterol and cholate production. 212.
  - cirrhosis, Co^{60} vitamin B_{12}. 681.
  - coproporphyrin excretion. 232.
  - cortisone metabolism. 27.
  - enzymes, pituitary growth hormone effect. 781.
  - glycogen, glycine, cortisone influence. 804.
  - hepatitis serum, immune serum globulin, safety. 554.
  - metabolism, sex difference, X-ray and fluoracetate effect. 695.
  - phosphorus metabolism. 771.
  - surgery, hypothermia. 639.
- **Lyxoflavin,** rations, starting. 395.

- **Malaria,** chicken tumor, inhibition. 349.
- **Mast cell,** hormonal influence. 335.
  - stilbamidine effect. 455.
- **Meninges,** talc granuloma, cortisone effect. 844.
- **Mercaptoethylamine,** X-ray lethality. 411.
- **Methylcholanthrene,** fibroma, formation. 34.
- **Mitochondria,** glycolytic and respiratory enzymes. 62.
- **Molybdenum,** toxicity, *Lactobacillus leichmannii.* 487.
- **Mouse,** C_3H, chloroform effect. 474.
- **Muscle** metabolism, vitamin E deficiency. 637.
- **Mustard,** influenza virus activity. 190.
  - nitrogen, growth hormone and antibiotic effects. 615.
    - toxicity, cortisone influence. 376.

- **Nerve** demyelination, seaweed. 518.
  - procaine and tetracaine effect. 831.
  - synapse, photography. 829.
- **Nitrogen** metabolism, testosterone effect. 674.
- **Nucleoprotein,** fibroblast stimulation. 390.

- **Oil,** sesame, DCA, sarcoma production. 14.
- **Osmosis,** membrane leaky. 195.
- **Ovary,** pituitary, gonadotrophic, hormone response. 157.
  - transplant, electroshock and electronarcosis. 78.
- **Oviduct,** amino acids and peptides. 697.

- **PABA** and PAS, oxidation. 378.
- **PVP,** intravenous, effects. 146.
- **Pancreas,** glucagon, pituitary growth hormone. 758.
  - pancreatitis, plasma coagulation. 423.
- **Paritol-C,** serum lipids and cholesterol. 665.
- **Peptides,** oviduct. 697.
  - -poly, glutamyl excretion. 253.
- **Phosphatide,** blood coagulation, X-ray. 384.
- **Phosphoramides,** tumor therapy. 438.
- **Phosphorus** metabolism, liver. 771.
- **Pimelic acid,** diamino, chromatography. 535.
- **Piromen,** X-radiation. 54.
- **Pituitary,** hematoporphyrin. 264.
  - removal, adrenal β-glucuronidase activity. 769.
  - tumors, thyrotrophin-secreting, thyroid hormone, effect. 511.
- **Placenta** transfer, vitamin A and carotene. 571.
- **Platelet extract,** intravenous, effect. 453.
- **Plutonium** distribution, zirconium effect. 710.
  - metabolism, Versene effect. 301.
- **Potassium,** deficiency, protein utilization. 102.
- **Proline** hydroxy, determination. 461.
- **Porphyrin,** proto, production, tetrahymena. 525.
- **Protein,** catabolism, hemorrhagic shock. 362.
  - utilization, mineral influence. 102.
- **Protozoa,** growth purines effect. 434.
- **Purine** 2,6-diamino, cell division and growth, inhibition. 561.
  - growth promotion. 434.
- **Pyrogen,** influenza, tolerance, influenzal virus fever production. 612.
  - mumps, tolerance, influenzal virus fever production. 612.

- **Radium,** effect, CF1 mice. 494.

Renin diuresis and proteinuria. 631.
Reproduction, X-ray effect. 662.
Rickettsia, Macacus infection. 699.

Schick test, cortisone effect. 502.
Schistosoma mansoni, castration influence. 83.
Seaweed, nerve demyelination. 518.
Sedormid, porphyria. 566.
Semen, fibrinolytic system relationship. 692.
Serum, "anemic," erythropoietic stimulation. 548.
Shock, hemorrhagic, fatty acid and ketone metabolism. 608.
    plasma aminopeptidase. 568.
    protein catabolism. 362.
Skin, temperature, measurement. 269.
Sodium retention, adrenal, venous blood. 628.
Soybean, soyin, immunology. 539.
Spleen, antineoplastic factor. 671.
Sprue, tropical, Co^{60} vitamin B_{12}. 681.
Starvation, intestinal motility. 98.
Sterilization, radioiodine, milk. 762.
Sterols, cholesterol C^{14} absorption. 498.
Stilbamidine, mast cell. 455.
Stomach, dye secretion, ionization constants. 130.
  secretion analysis, tubeless, exogenous ions. 483.
    vitamin B_{12}, electrophoresis. 506.
  vitamin B_{12} absorption. 226.
Streptokinase, antigen. 689.
Stress, body temperature. 4.
  reducing factors. 135.
  serum, electrolytes and electrocardiogram. 660.
  sulfhydryl compounds, blood and liver. 708.
  wound healing. 798.
Strontium 89 metabolism, chemical agent, effect. 550.
Sulfhydryl, blood and liver, "stress" effect. 708.
  tyrosinase and protyrosinase. 717.
Sulfonamide, growth effect. 88.

Teeth, caries, experimental, skim milk. 21.
Telepaque, excretion. 544.
Temperature, body, stress, anesthesia, death effect. 4.
Testicle, atrophy, radiation induced, pituitary extract effect. 259.
Tetrahymena, folic acid analogs, effect. 586.
Thiopental, anesthesia, fat feeding effect. 110.
Thromboplastin, thromboplastinase. 858.
Thyroid, cholesterol metabolism. 143.
  cholic acid and cholesterol excretion. 246.
  I^{131}, uptake, food and water effect. 748.
  iodine utilization, fish. 751.
  pituitary axis, cortisone effect. 181.
  struma lymphomatosa, experimental production. 835.
Thyronine, iodine metabolism. 52.
Thyroxine, iodine metabolism. 52.
  thyroid effect. 875.
  tissue metabolism. 500.
Tissue clearance. 643.
  diet, cattle fed dot-dusted alfalfa hay. 458.
Toluidine blue, metastatic calcification. 281.
Triazines, 1,2-dihydro-s-. 733, 740, 742.
Tumor, anti, splenic factor. 671.
  ascites, characteristics. 520.
  chicken, malaria effect. 349.
  experimental, salivary gland. 414.
  fibroma, methylcholanthrene. 34.
  Flexner-Jobling, phytoagglutinins. 863.
  sarcoma, DCA, sesame oil injection. 14.
  therapy, phosphoramides. 438.
Thyronine, triiodo-, thyroid effect. 875.

Urine porphyrin, sedormid. 566.
  protein and renin diuresis. 631.
Uterus, acid phosphatase. 45.
  glycogen deposition, starvation. 405.

Vanadium, Versene, antagonism. 346.
Versene, plutonium, metabolism. 301.
  vanadium, toxicity. 346.
Virus, arthropod-borne, hemagglutinins. 170.
  Coxsackie, properties. 119.
  encephalitis, St. Louis, propagation in tumor. 822.
  growth kinetics, tissue culture. 621.
  herpes zoster, propagation. 340.
  infectious bronchitis, polypeptide protection. 641.
  influenza, fever production. 612.
    mustard effects. 190.
  Mack. 200.
  mouse pox, spontaneous. 315.
  mumps, hamsters. 714.
    polypeptide effect. 852.
  Newcastle disease, polypeptide protection. 641.
  poliomyelitis, antibiotic. 161.
    benzimidazole effect. 408.
    cytopathogenic inhibition, antibiotics. 787.
    erythrocyte absorption. 249.
    fatigue, effect. 678.
    gamma globulin effect. 308.
    monkey, oral infection. 807.
    tissue culture, susceptibility. 577.
    type III, mouse adapted. 706.
  psittacosis, turkey. 99.
  rabies, Syrian hamster. 793.
  vaccinia, plaque formation. 426.
    protection, egg cultivated tumor. 676.
  varicella, propagation. 340.
  whooping cough, antibodies. 866.
Virus-anti, netropsin. 1.
Vitamin A, deficiency, oral epithelium, phase microscopy. 356.
    placenta and mammary transfer. 571.
    utilization, aureomycin effect. 43.
  B_1 deficiency, cortisone effect. 872.
  B_6 deficiency, eosinopenile, adrenal and stress. 655.
  B_{12} assay, *E. coli*. 448.
    Co 60 retention. 221.
      tropical sprue and hepatic cirrhosis. 681.
    excretion, gastrectomy, influence. 226.
    gastric secretion, electrophoresis. 506.
  C, biosynthesis, precursor. 39.
    deficiency, 17-hydroxycorticosteroids. 722.
  citrovorum factor, metabolism. 167.
  E, deficiency, arginase. 57.
      muscle metabolism, nitrogen. 637.
  survival, eviscerated rat. 602.
  thiamine, excretion, mercurial effect. 323.

Vitamin-anti, amethopterin, tissue. 369.
  folic acid analogs, tetrahymena. 586.
    resistance. 272.
  pteroylglutamic acid, metabolism. 167.

Wound healing, stress effect. 798.
  sulfur amino acids. 329.

X-radiation, antibody production. 163.
  β mercaptoethylamine protection. 411.
  bactericidal action, inhibition. 184.
  carboxypeptidase inhibitor. 6.
  infection, susceptibility. 205.
  life prolongation, dialysis *in vivo*. 618.
  live metabolism, sex difference. 695.
  piromen effect. 54.
  reproduction paramecium. 662.
  somatotrophic hormone and streptomycin influence. 85.
  streptomycin, host defenses. 289.
  tissue enzymes. 266.
X-ray, coagulation defect, phosphatide effect. 384.

Yeast cultivation, mouse cells. 584.

Zinc, cytochrome oxidase. 625.
Zirconium, plutonium distribution. 710.